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Are You What You Eat? Selective Sequestration of Toxic Milkweed Cardenolides in the Monarch Butterfly

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Are You What You Eat?

Selective Sequestration of Toxic Milkweed Cardenolides

in the Monarch Butterfly

A thesis submitted in partial fulfillment of the requirement
for the degree of Bachelor of Science in Biology from
The College of William and Mary

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Abstract

The Monarch butterfly, *Danaus plexippus*, is projected to face substantial quasi-extinction risk over the next 20 years after decades of population decline (Semmens et al., 2016). The butterfly shares an exciting and oft-studied chemical relationship with its larval food source, milkweed. Monarchs have the ability not only to tolerate the plant's toxic cardenolide defenses, but also to sequester them into their own tissue for defense against predators and parasites. Recent work demonstrating a strong correlation between the cardenolides of the milkweed host and the eventual parasite load of the metamorphosed adult motivated us to further investigate the passage of defenses from plant to herbivore (De Roode et al., 2016). Here we perform a feeding trial with seven unique genets of Common milkweed (*Asclepias syriaca*) and collect samples of Monarch larvae, pupae, adults and frass. We compare these samples' cardenolide chemistry to that of their host plants using high-performance liquid chromatography. We find that cardenolide concentration varies significantly between *D. plexippus* and *A. syriaca* but not between the individual tissue types mentioned above. Our seven milkweed genets were obtained either from sites in North Carolina or Virginia and this geographic variation did not affect variation in cardenolide concentration. We find that cardenolide concentration across all samples varies over time throughout the experiment. We show that some cardenolides identified are substantially more concentrated than others and that some appear only in milkweed or Monarch tissue. We find the relative concentrations of several most prominent cardenolides to vary significantly between plant and insect samples. We show that cardenolide profiles in Monarchs vary significantly from those in milkweed and vary significantly over time. We demonstrate that insects exercise substantial discrimination in their cardenolide sequestration and we show that this sequestered profile is stable throughout their development. Ultimately, replication and a larger dataset are needed to draw finer conclusions about differences in cardenolide content between milkweed, Monarchs, and their respective tissues.

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Introduction

Following a fifteen-year trend, the North American population of the Monarch butterfly, *Danaus plexippus*, has continued to experience decline (Semmens et al., 2016, Pleasants et al., 2016). A model for the quasi-extinction risk in the Monarch has predicted an 11-57% risk over the next twenty years, with a five-fold increase in population necessary to halve that likelihood (Semmens et al. 2016). Unique Monarch migratory behavior has been termed an "endangered biological phenomenon" and despite year-to-year fluctuation, the net trend remains negative and the outlook for the species is clouded by pessimism and concern (Brower et al. 2011).

Common milkweed, *Ascelpias syriaca,* is native to North America and ranges across the eastern and central United States and Canada, east of the Rocky Mountains (Agrawal, 2005). The perennial, whose ramets grow from a rhizome, prefers edge habitats and sandy soils (Agrawal, 2005). Milkweeds, in keeping with a theme established by plants all over the planet, synthesize irritating

defensive chemicals to prevent their herbivory by insects. One of these, latex, has long received attention in the literature and has been used both as a metric of plant health and as a predictor of energetic investment on the part of the plant (Herms and Mattson, 1992). The noteworthy secondary metabolite that forms the basis of the following investigation, however, is the cardiac glycoside (CG) or cardenolide.

Cardenolides are steroid structures with toxicity enabled by their binding affinity for the Na^+/K^+-ATPase pump (Petschenka and Agrawal, 2015; Figure 1). Association of CGs with their binding site on the pump inhibits its activity (Dalla

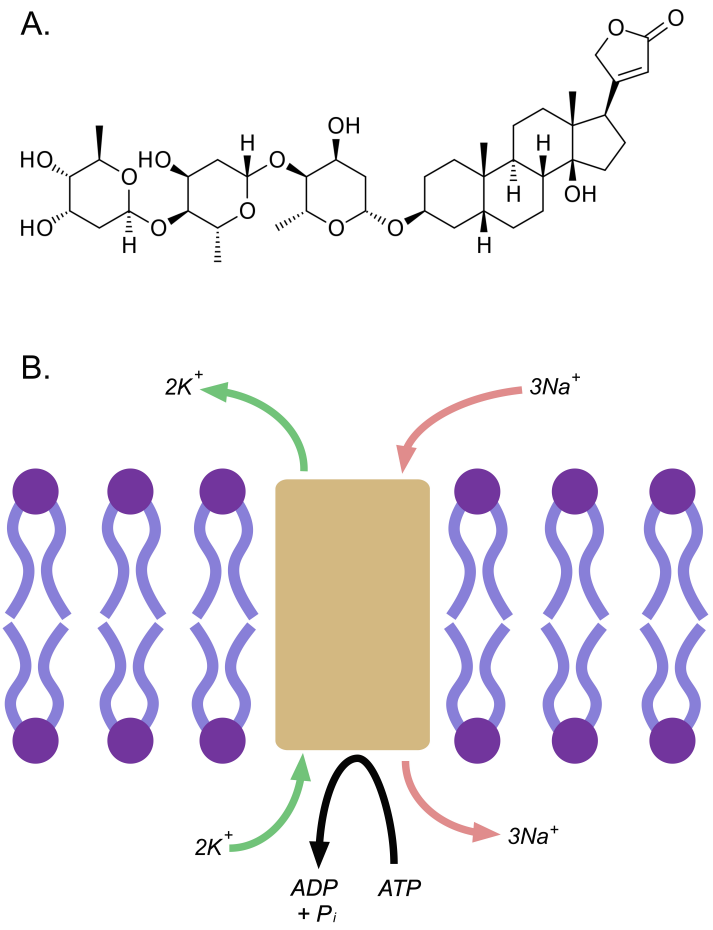


Figure 1: **A.** Digitoxin ($C_{41}H_{64}O_{13}$; 765 g/mol), our internal standard in HPLC, is a cardiac glycoside. **B.** The Sodium-Potassium exchanger enzyme maintains a low intracellular concentration of Na^+ and a high intracellular concentration of K^+, pumping both against their concentration gradients.

et al., 2013). The antiporter is responsible for the ion concentration gradients that initiate muscle contraction. Hindrance of this cation transport results in paralysis and death by suffocation (Dalla et al., 2013). High conservation of this protein means the sensitivity of this pump can be observed in arthropods and large vertebrates alike. A resistance to cardenolides' toxic effects is believed to have

evolved independently in five herbivorous insect taxa and in a stepwise manner within the *Danaini* (Petschenka and Agrawal 2015). Three derived pumps with increasing degrees of CG-resistance conferred by site-specific amino acid substitutions can be seen in *Danaus*, with the most robust resistance appearing in the Monarch's own enzyme (Petschenka and Agrawal 2015).

At face value, insect herbivore tolerance of plant defenses seems a classical example of an "coevolutionary arms race", but recent work suggests a more compelling and nuanced scenario (Agrawal et al.,2016; De Roode et al., 2016). The Monarch is not only able to tolerate milkweed defensive poisons but also able to incorporate them into its own tissue (Dalla et al., 2013). This phenomenon has become widely researched and generated great interest. CG sequestration is of great ecological significance as cardenolide-rich milkweed substrate has been shown to considerably reduce parasite load in adult butterflies (De Roode et al. 2008). This adds a tritrophic wrinkle to the plant-herbivore relationship and represents a crucial ecological consequence for the offspring of that adult butterfly amid the evolutionary interest of sequestration. The spores of a particular parasite, *Ophryocystis elektroscirrha*, are transmitted to the adult female's eggs during oviposition. A reduced parasite population in the mother's body reduces the likelihood of reinfecting her offspring (De Roode et al. 2011).

The astonishing migration of the Monarch entails about 4,830 miles and has been called "one of the most spectacular natural phenomena in the world" (Brower, 1977; Dingle, 2014). It takes about four generations to complete the trip and along the way *D. plexippus* larvae feed on several species of the milkweed genus, *Asclepias*.

Of the genus' 108 species, the Monarch is known to feed on 27 (Malcolm and Brower 1989).

It has been noted that cardenolide sequestration activity varies as a function of the *Asclepias* species Monarchs were reared on (Malcolm and Brower 1989). *A. syriaca* is not only a colossally important food source for migratory Monarchs: *D. plexippus* has also been shown to sequester cardenolides sourced from *A. syriaca* more effectively than those from any other member of *Aslcepias* (Malcolm and Brower, 1989). The possession of different cardenolides at varying concentrations in *Asclepias* species has been correlated with the spatial distribution of their ranges across North America (Rasmann and Agrawal, 2011). We hypothesize that the resulting toxicity of Monarch tissue may vary between individual host plants of the same species if those host plants represent geographically different populations. This consideration is especially salient in light of the observation that cardenolide concentration and diversity within *Asclepias* have been shown to vary latitudinally, with milkweed defenses most robust at lower latitudes (Rasmann and Agrawal, 2011). After oviposition, the generation to embark on the next leg of the journey will be defended by chemicals obtained by their mother from a milkweed plant that may be hundreds of miles away. Indeed, they may be dependent for their defense from parasites upon a milkweed species that is not endogenous to the latitudes at which they will live their entire lives. The toxicity of that host plant will have reflected its environment, not theirs or that of the milkweed they encounter.

We used high-performance liquid chromatography to identify and quantify CGs in an experiment that sought to answer several questions dealing with the Monarch's relationship to its host plant defenses. The first, and simplest: how closely does the Monarch's cardenolide content match that of its host plant? This straightforward consideration is made more interesting in light of the observation that milkweed chemical defenses, including cardenolides, may be induced under conditions of herbivory (Wang et al., 2014). The experiment entails measurements of leaf tissue chemistry over a period of several weeks. Plant and insect cardenolides may be compared over time in light of fluctuations in the plant's toxicity. This gives us the opportunity to observe any corresponding changes in that of the insect.

It has been reported in the literature that insects are observed to contain certain CGs and not others (Malcolm et al., 1989). We sought to record this, asking, if not all CGs are transmitted from plant to herbivore, which ones are and in what proportions? Are there any unifying characteristics of the sequestered CGs?

Additionally, we anticipated that the conversion machinery responsible for observed discrepancies in the concentrations of *individual* cardenolides between the two tissues might generate new peaks in the insect tissue chromatogram, not observed in the plant (Malcolm and Brower, 1989). To this end, we wondered: are any novel cardenolides observed in larval, pupal or adult insect tissue that cannot be found in its larval food plant?

Finally, an experiment with measurements tracking the CG profile over time gives us the opportunity to ask an important question and one that has bearing on the conclusions of all the previous. Is a Monarch's cardenolide profile stable

throughout its development, or does it change over time? We wondered if maintenance or dynamism of the insect's cardenolide content might reflect the wildly different ecological interactions of its larval, pupal and adult life history windows.

This project was influenced in many respects by the findings of Malcolm et al., 1987. In that paper, the researchers use spectrophotometry to measure the total cardenolide concentration of wild *A. syriaca* and *D. plexippus* samples and thin layer chromatography (TLC) to determine *A. syriaca's* 'cardenolide fingerpring' (what we refer to here as a 'cardenolide profile'). The use of a spectrophotometer limited their spectroassay to a measurement of total cardenolide concentration and their differentiation of unique cardenolides by TLC has acknowledged weaknesses. TLC results may be complicated by the interaction of pregnane glycosides with the detection reagent, TNDP or by the rapid fading of TNDP's blue reporter color (Malcolm et al., 1987). High performance liquid chromatography (HPLC) has superior resolution to TLC and gives the ability to qualitatively distinguish peaks and simultaneous quantify their concentration with high precision (Kautsky, 1981). However, other noncardenolide chemicals likely present at 220nm in HPLC of tissue extract, so it is possible for these compounds to present as cardenolides and interfere with results (Kautsky, 1981). We address this concern with rigorous spectral interpretation criteria that may exclude some extremely low-concentration cardenolides but drastically improves our confidence in the identity of all others. Insect and plant tissue samples in the 1987 publication were prepared with separate extraction protocols, in ours the same was used for both.

Methods

In the spring and summer of 2015, a series of feeding trials were conducted attempting to quantify the energetic cost of cardenolide sequestration in several species of milkweed-specialist insects. The growth of Monarch larvae, as well as Milkweed bugs (*Oncopeltes fasciatus*) and Longhorn milkweed beetles (*Tetraopes tetrophthalmus*), was observed and recorded over time in an attempt to establish a connection between the rate of development and the toxicity of feeding substrate. We did not identify such a relationship and, having observed a trend in the literature, determined feeding trials to be an inconsistent and unreliable method to examine this phenomenon. The results of these investigations led us to consider a more refined approach to the identification and quantification of plant cardenolides within herbivore tissue.

Experimental Design

Seven unique milkweed genets, half obtained from the North Carolina Botanical Garden and half collected by Cyrus Brame from Presquile Wildlife Refuge in Virginia, were used as feeding substrate for the development of 100 Monarch larvae. To produce sufficient food stock for the entire population of insects, four to five cuttings from each genet were cloned and potted separately in Farfard 4P. The rhizome cuttings were all obtained from a single individual of each genet to ensure genetic identity.

Larvae were obtained from Shady Oaks Butterfly Farm in Brooker, Florida. Eight to twelve first instar larvae were devoted to each genet and were reared on cut leaves through the second instar until they were large enough to be easily monitored on a potted, netted plant. By the second instar, the larvae were transitioned to the netted plants from which their feeding substrate had been sourced until that time. All larvae were allowed to eat freely and develop within enclosures that were cleaned and sterilized daily. Throughout the experiment, plant or insect tissue samples were frozen at -80°C before extraction and analysis.

Insect tissue samples were collected from each genet-specific population at the larval, pupal and adult stages. In parallel, leaf tissue samples were collected from each genet on the same day. The solid waste of the developing larvae was also collected and frozen as they progressed through their five instars (Figure 2). Before freezing, sacrificed larvae were removed from food for 24 hours before freezing to allow time for the passage of any leaf material in the gut.

The first of these samples were leaves gathered at the same time as daily feeding stock for the newly hatched larvae. These reflected the experiment's ‘t=0’ and established the baseline cardenolide concentrations of each genet before putative defense induction. The first insect samples collected were third instar individuals. Over the course of the Monarch’s two weeks of development, a larva was collected and preserved as often as possible at noon each day. Larval collection was constrained by the need to leave some individuals for pupation. Likewise, pupa collection was constrained by the need to allow some individuals to emerge as

metamorphosed adults. Whenever insect tissue was preserved, tissue from the ramet on which that individual had been feeding was preserved as well.

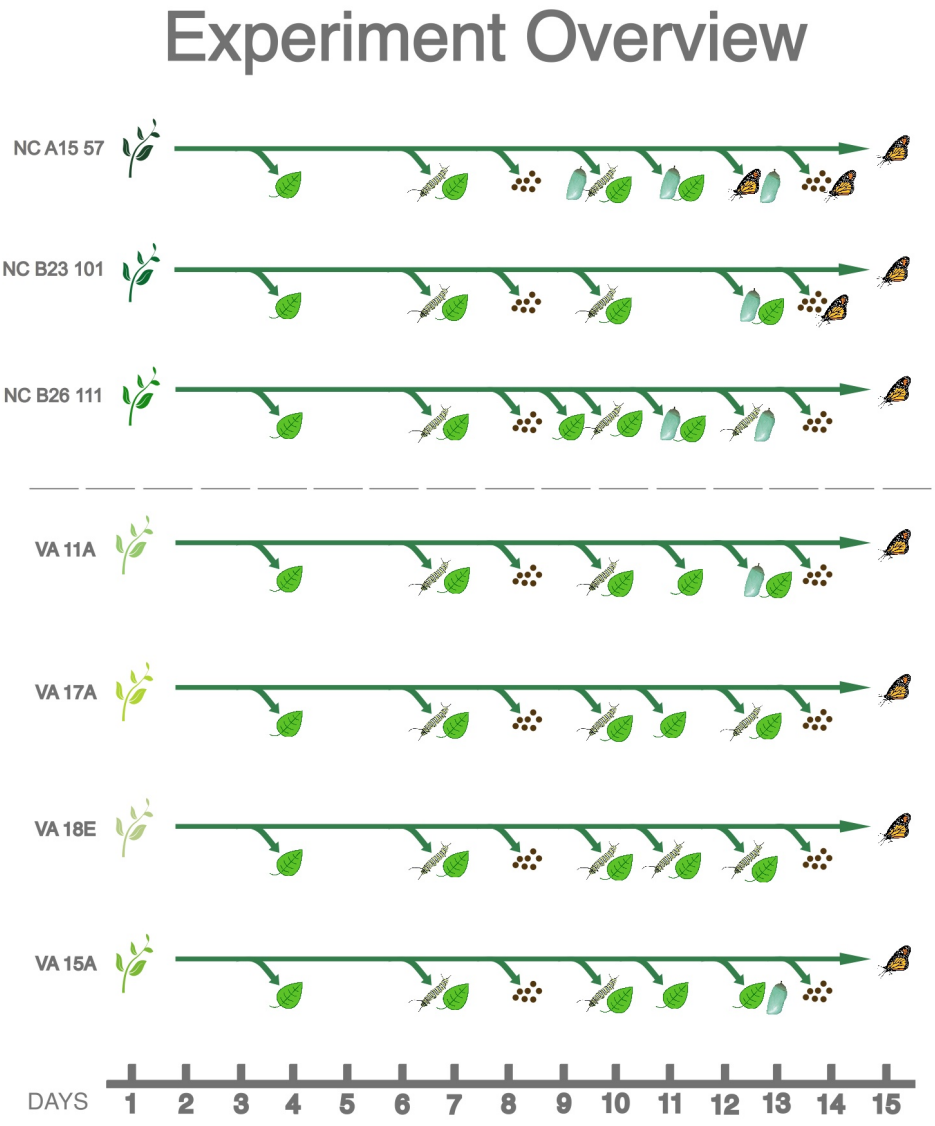


Figure 2: The workflow of the experiment, representing the days on which certain tissues were collected from particular milkweed genets from both locations. Each row describes one of seven genets on which Monarchs were reared and is labeled with the name of that genet on the left. Cartoon representations of each of the tissues collected (leaf, larva, frass, pupa, adult) are pictured at the day they were obtained.

Extraction Protocol

Tissue samples, frozen in 15mL falcon tubes were lyophilized for 24 hours. About 50mg (+/- 5mg) of freeze-dried tissue (exact weights for each sample were

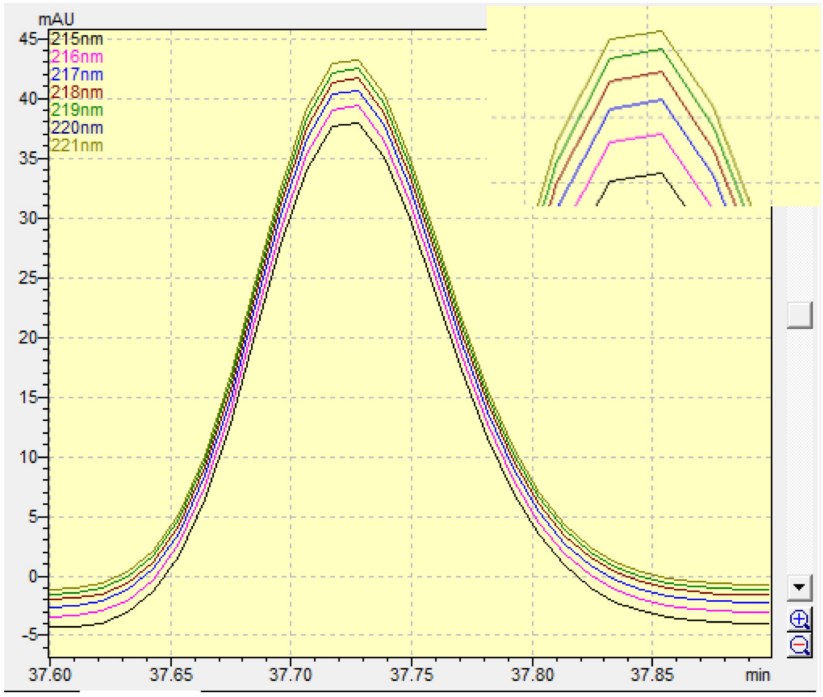
recorded) was transferred to a bead ruptor tube with three steel beads. Dry tissue was combined with a 100% ethanol buffer and 20μL of a digitoxin standard of known concentration. Tissue was first homogenized on a ball mill for three 60-second intervals with 60-second rests in between and then in a room temperature bath sonicator for 20 minutes. Matrix tubes were left open and incubated for twelve hours at 50°C to evaporate all ethanol. Samples were then reconstituted within the bead ruptor tubes in 800μL of methanol. While some similar procedures in the literature treat milkweed and Monarch samples with separate extraction protocols, we use the above protocol to homogenize samples from both species.

HPLC Protocol

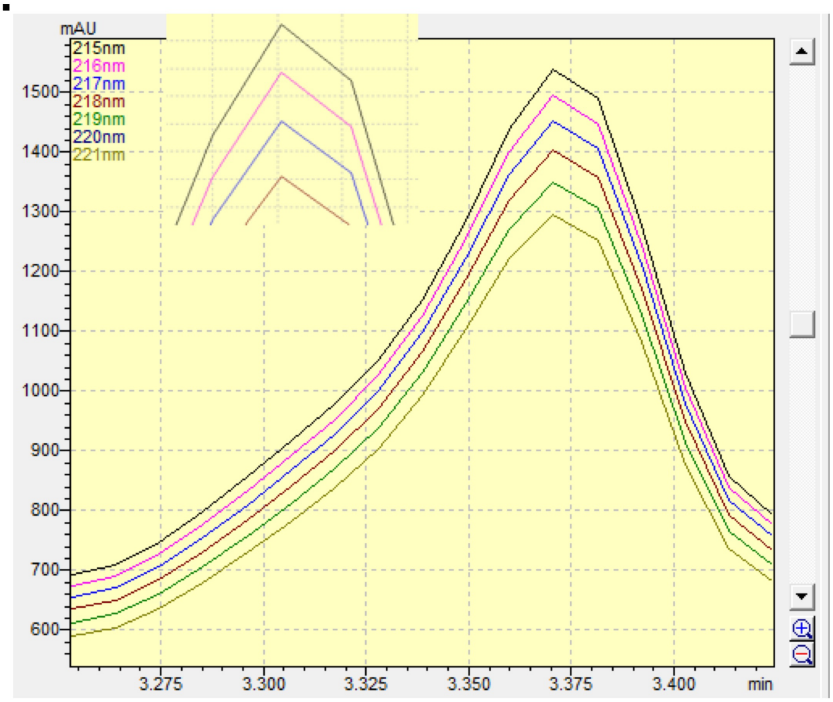
For HPLC, 500μL of each sample was transferred from the ruptor tubes into whatman vials of which 15μL was injected into the device. Every operation of the instrument included a methanol blank (500μL of methanol) at the beginning and at the end of the run. Each run also included an extraction blank (500μL of 100% ethanol and 20μL of digitoxin standard) that underwent the aforementioned tissue homogenization protocol. Data was gathered with a Shimadzu Prominence instrument and all interpretation was performed within the associated LCsolution software. A variable composition solvent mix of acetonitrile and a 0.25% phosphate buffer was used. A new liter of buffer was prepared for each run of the machine. Each sample was passed through the column for 55.1 minutes at a constant flow rate of 0.7 mL/min. The mobile phase began each run as a 20% acetonitrile/80% phosphoric acid mixture. After 5 minutes this composition was shifted to 70/30%

for 20 minutes. Then a 95/5% composition finished the remaining 30 minutes of the run.

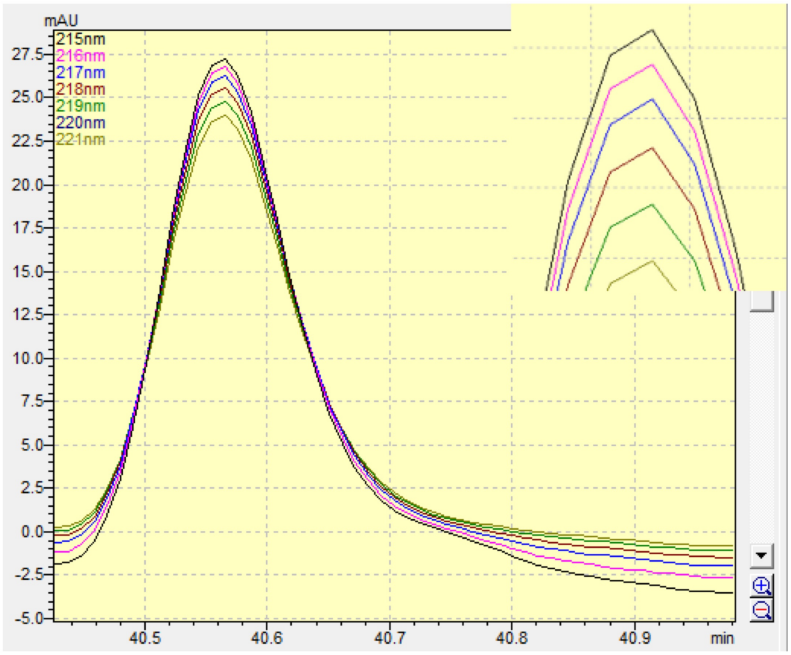
A.



B.



C.



D.

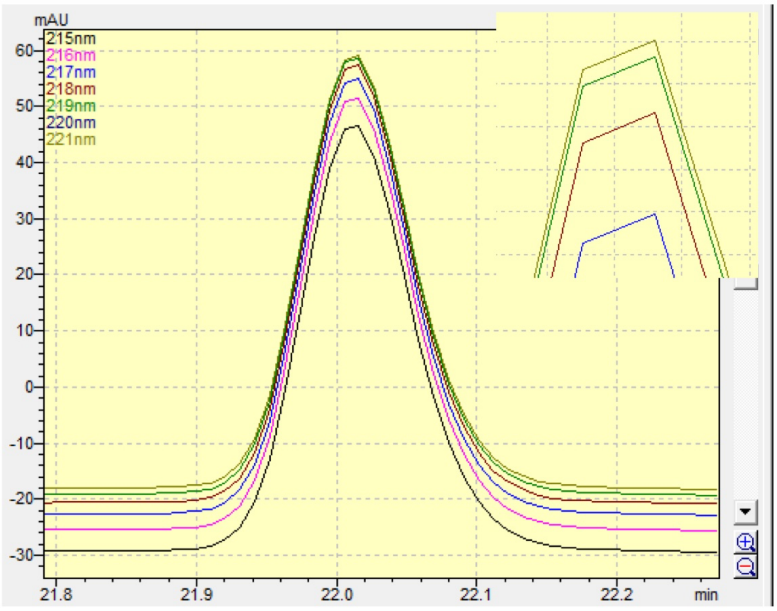


Figure 3: A. A cardenolide peak appearing in a larval sample reared on NC.A15.57 for 7 days. The peak is symmetrical, has a base width of about 0.5 minutes and maximum absorbances of 221nm, 219nm, and 218nm (inset). **B.** An example of an asymmetrical peak with the wrong maximum absorbance wavelengths (inset). This peak can easily be ruled out. **C.** An example of a symmetrical peak without the correct maximum absorbance wavelengths (inset). This peak superficially resembles a cardenolide, but can nonetheless be omitted from consideration. **D.** An example of digitoxin, a known cardenolide and the internal standard for all HPLC data. Note the characteristic peak symmetry and maximum absorbance at 221, 219 and 218nm.

Spectra were generated using an SPD-20A detector module. The module contains a diode array detector suitable for the identification of our target compounds, which emit at UV wavelengths. UV absorbance spectra were recorded between 200 and 400 nm. Peaks were recorded as cardenolides on the basis of several criteria reported and employed in the literature. Highly symmetrical peaks with maximum absorbances at 218, 219 and 221 nm and with base width of about 0.5 minutes were considered cardenolides (Agrawal 2014, Rasmann et al., 2011, Rasmann et al., 2009, Wiegrebe and Wichtl, 1992; Figure 3).

The concentration of each peak was determined by comparison with the area under the curve and known concentration of the internal digitoxin standard from each sample. With a known concentration of added digitoxin and with a known sample dry weight it was possible to calculate the concentration of digitoxin in each sample. Afterwards, the concentration of each identified cardenolide could be determine by a simple ratio of its peak's area to that of the internal standard in that sample. Digitoxin elutes from the column at about 22.0 minutes. The fluctuation in its absolute retention time is usually quite small (.0001-.001 minutes) but it was nonetheless necessary to control for this slight variability between samples so they could be meaningfully compared. All retention times are reported relative to the digitoxin standard in their sample. These 'relative retention times' can be multiplied by 22.0 to give the approximate number of minutes it took for the compound to elute from the column.

Results

Variation in the Most Common Cardenolides

We performed a principal component analysis (PCA) of the five most common cardenolides observed in all samples (relative retention times 1.05, 1.2, 1.55, 1.7, 1.85). The first principal component resulting from analysis of these five retention times was shown to vary significantly between *A. syriaca* and *D. plexippus* samples ($t(50)=6.13$, $p<0.0001$). The variation in toxicity between plant and insect tissue samples is well explained by principal component 1 (Figure 4a). Generally speaking, cardenolides appear at higher concentrations in plant tissue than in insect tissue ($t(227)=5.10$, $p<0.0001$).

Clustering of the principal components does not suggest that the variation in cardenolide concentration can be explained by the geographic origin of the samples (Figure 4b). However, differences between North Carolina or Virginia plant origin were important to principle component 1 but not to principle component 2 ($t(49)=1.68$, $p=0.0494$; $t(56)=0.62$, $p=0.269$; respectively).

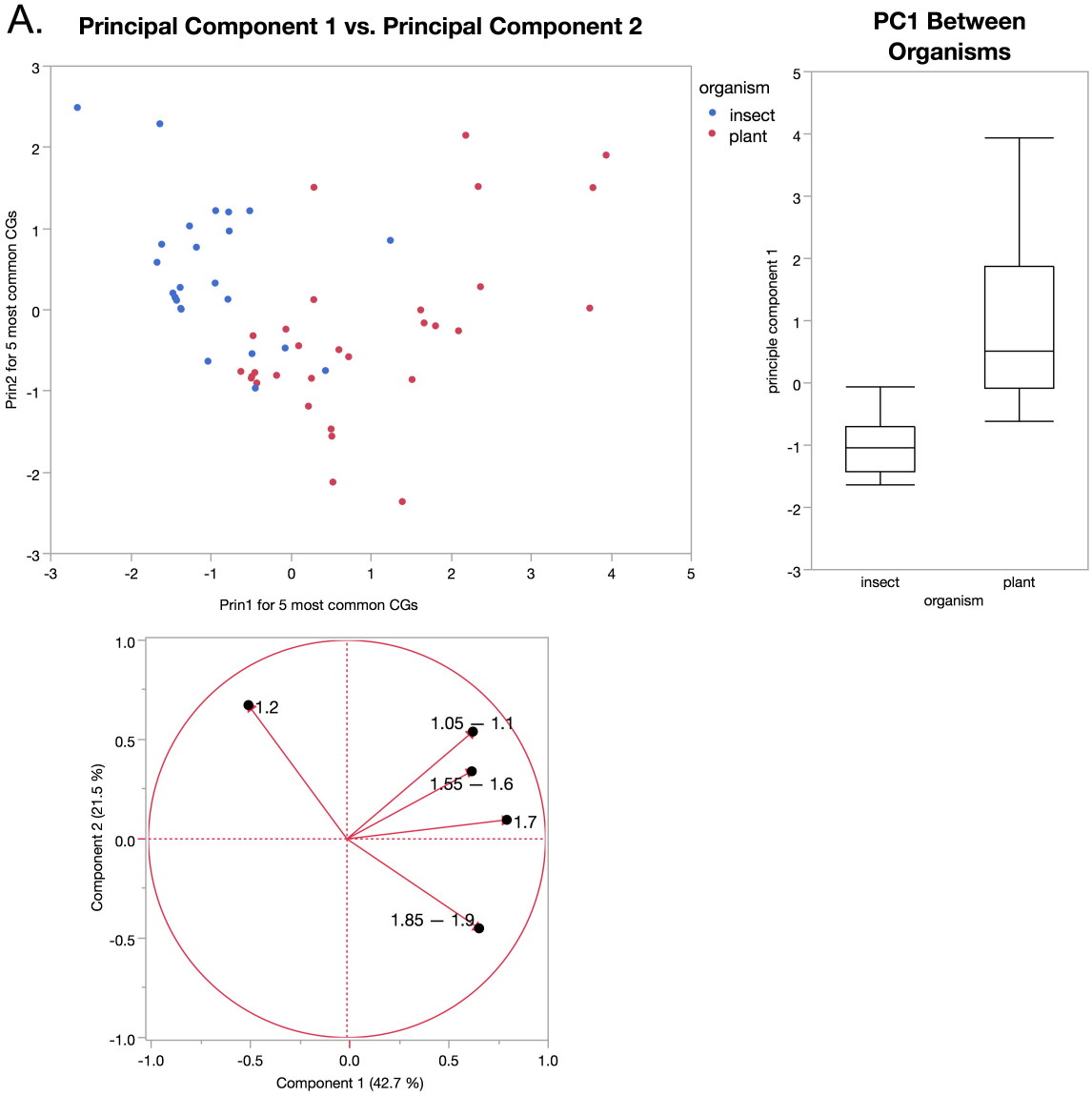
We investigated the importance of North Carolina or Virginia origin to separate groups of insect and plant samples. Among insect samples, no clustering of principal component values is visible when colored by origin and the values of principal component 1 did not differ between origin groups ($t(19)=0.64$, $p=0.73$; Figure 5a). No difference between origins was observed in actual concentration values from insect samples ($t(55)=0.079$, $p=0.46$; Figure 5a) Within plant samples, a similar lack of clustering was observed but the values of principal component 1 did show substantial differences between origins ($t(28)=1.86$, $p=0.036$; Figure 5b).

Comparison of actual concentration values between North Carolina and Virginia plant samples revealed no differences, however ($t(113)=0.57$, $p=0.715$; Figure 5b).

The genet from which the tissue had come, either directly or through feeding, did not explain the variation in cardenolide concentrations (Figure 6). Individual *syriaca* lines did not significantly affect toxicity variation in their leaves or in the insects that fed on them ($F(6, 58)=1.14$, $p=0.35$).

While varying with respect to organism, principal component 1 was also shown to vary significantly over time. Analysis of variance revealed substantial differences in the value of principal component 1 between 'time windows' and between discrete days ($F(3, 58)=12.18$, $p<0.0001$); $F(10, 58)=3.71$, $p=0.0010$); respectively; Figure 7). The time windows – "early", "mid", "late" and "very late" – comprise approximately equal spans of time during the two-week development of the Monarch and account for inconsistencies in the collection of samples on each individual day. *D. plexippus* samples showed variation in principal component 1 across time windows while *A. syriaca* samples did not ($F(2, 22)=10.05$, $p=0.0009$; $F(3, 29)=1.85$, $p=0.1628$; Figure 7a,b; respectively).

A.


Principal Component 1 vs. Principal Component 2
Prin2 for 5 most common CGs
Prin1 for 5 most common CGs
organism
insect
plant
PC1 Between Organisms
principle component 1
insect
plant
organism
1.2
1.05 — 1.1
1.55 — 1.6
1.7
1.85 — 1.9
Component 2 (21.5 %)
Component 1 (42.7 %)

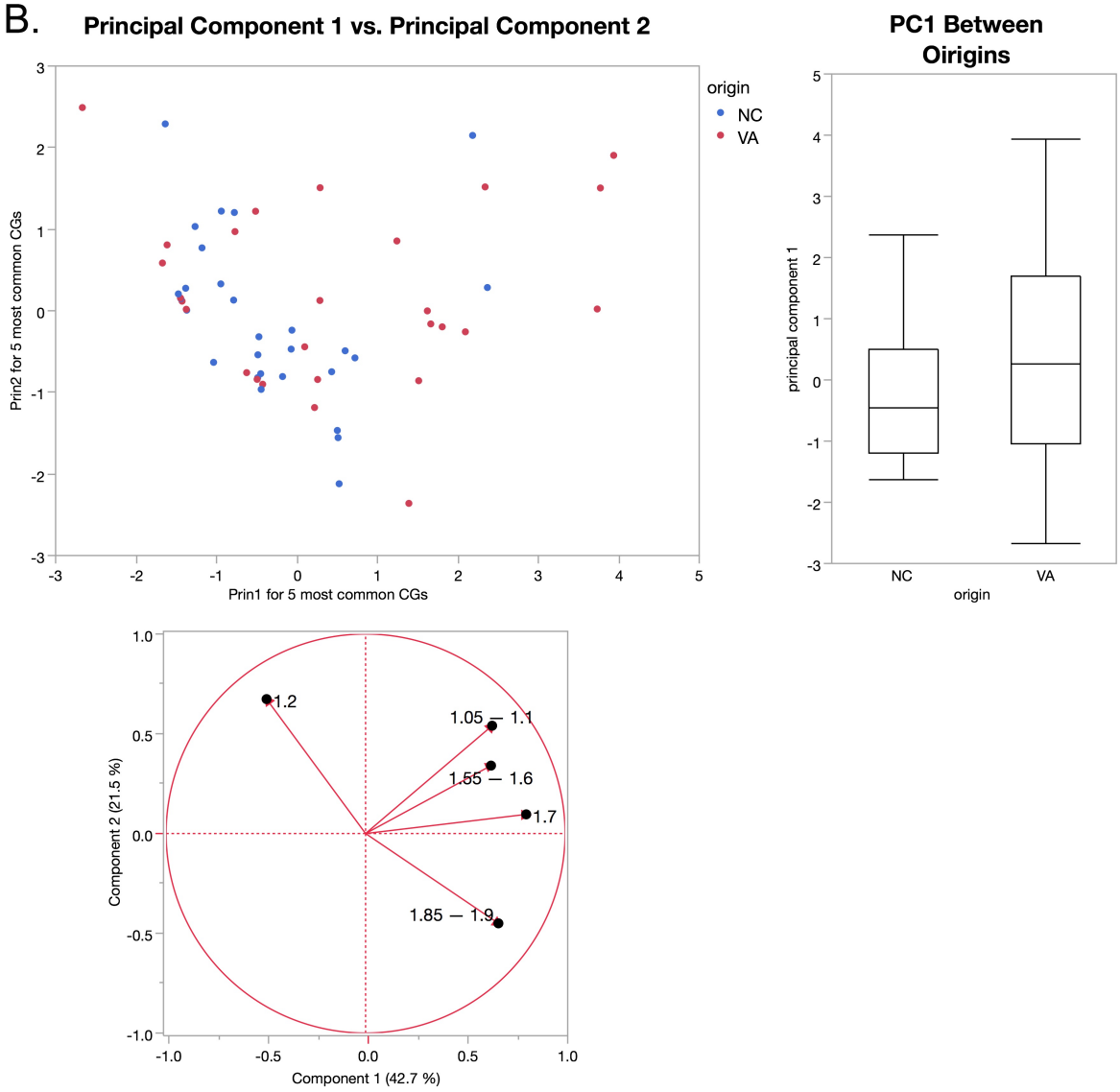


Figure 4: A. A scatter plot of principal components 1 and 2 explaining variation in cardenolide concentration values. Distinct clustering of these values is visible for insect (blue) and plant (red) samples. The difference between insects and plants accounts for the variation explained by PC1 ($t(50)=6.13$, $p<0.0001$). **B.** A scatter plot of principal components 1 and 2 using the same loadings. Cardenolide concentration values are shown not to cluster by geographic origin. PC1 is explained by origin ($t(49)=1.68$, $p=0.0494$).

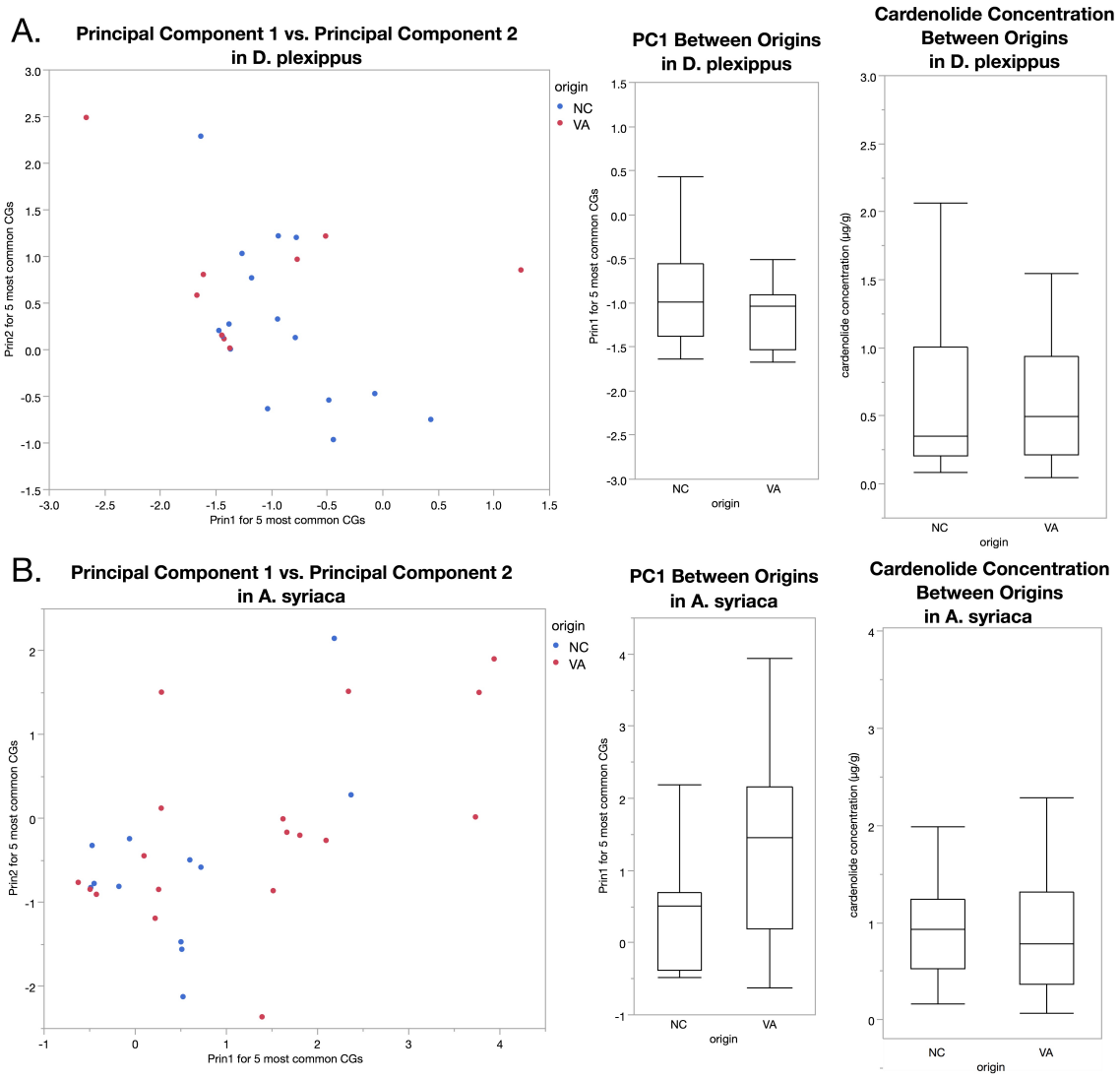


Figure 5: A. (Left to right) A scatterplot of the same principal components in only insect samples. A boxpot of principal component 1 between origins in only insects. No difference was observed between PC1 values for each origin ($t(19)=0.64$, $p=0.73$). A boxplot of actual concentration values between origins for insect samples. No difference was observed between origins ($t(55)=0.079$, $p=0.46$). **B.** (Left to right) A scatterplot of the same principal components in only plant samples. A boxplot of principal component 1 between origins in only plants. The values of PC1 vary significantly between origins ($t(28)=1.86$, $p=0.036$). A boxplot of actual concentration values between origins for plant samples. No significant difference was observed between origins ($t(113)=0.57$, $p=0.715$).

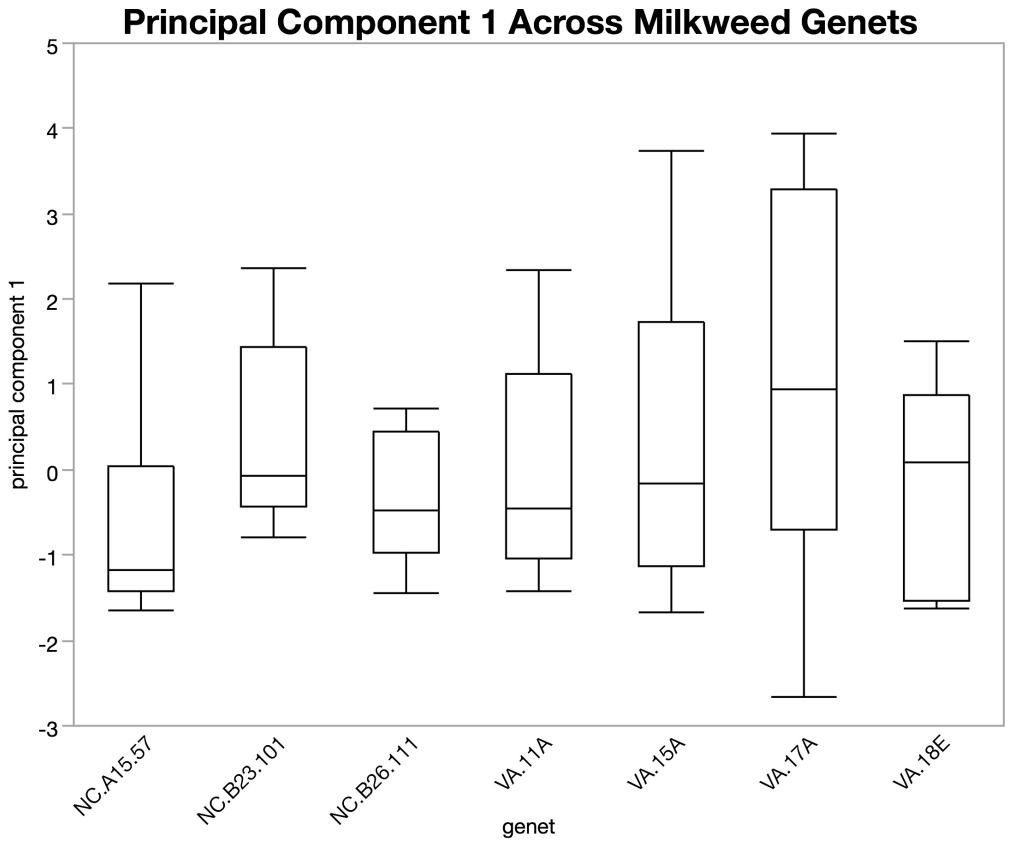
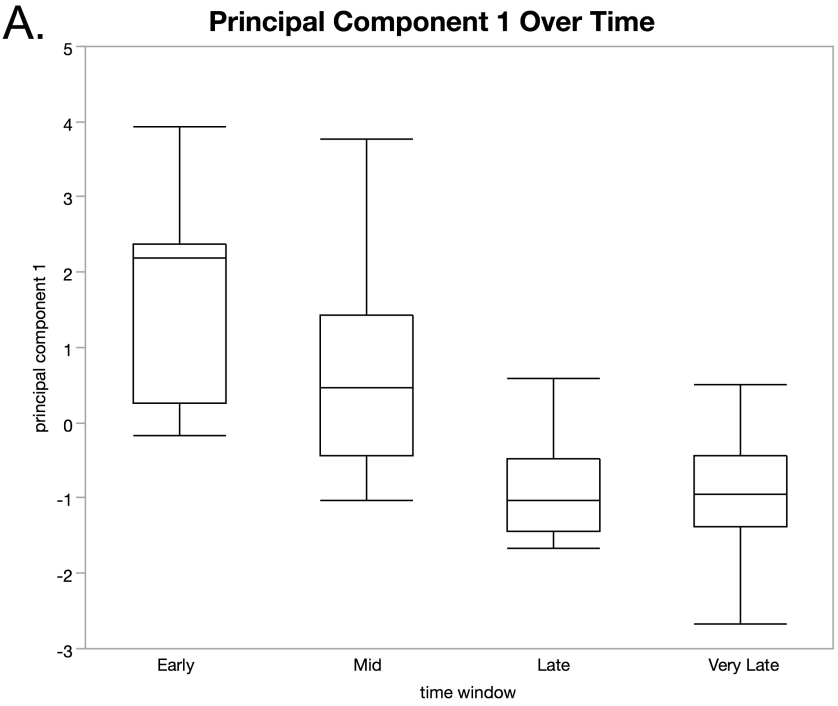
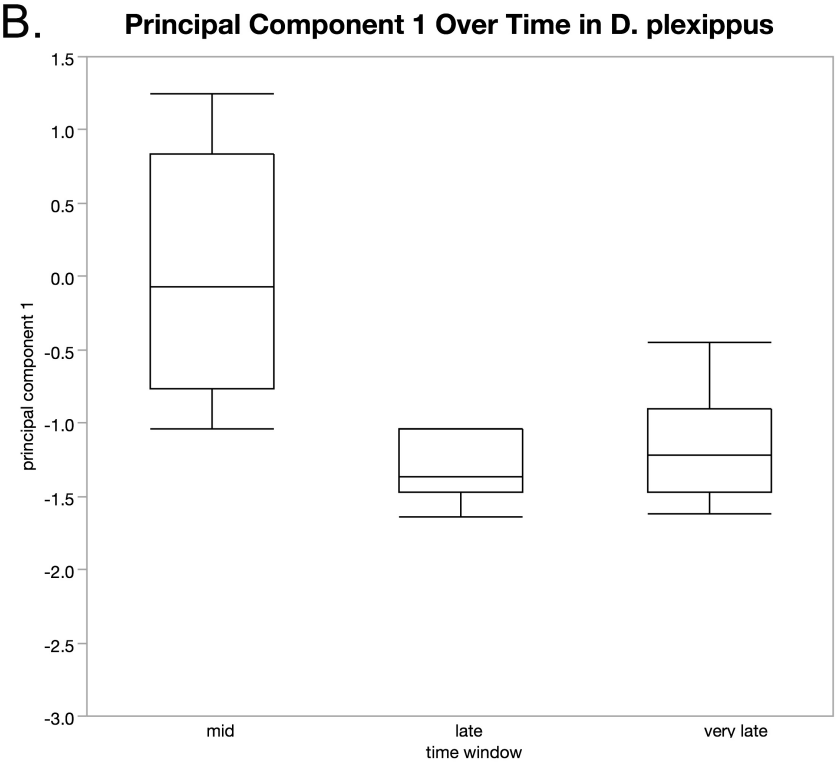


Figure 6: Variation in the values of principal component 1 across the seven milkweed genets did not meet our significance threshold. Differences between these genets do not explain the variety in the data accounted for by this principal component 1 ($F(6, 58)=1.14$, $p=0.35$).

A.


Principal Component 1 Over Time
principal component 1
5
4
3
2
1
0
-1
-2
-3
Early
Mid
Late
Very Late
time window

B.


Principal Component 1 Over Time in D. plexippus
principal component 1
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
mid
late
very late
time window

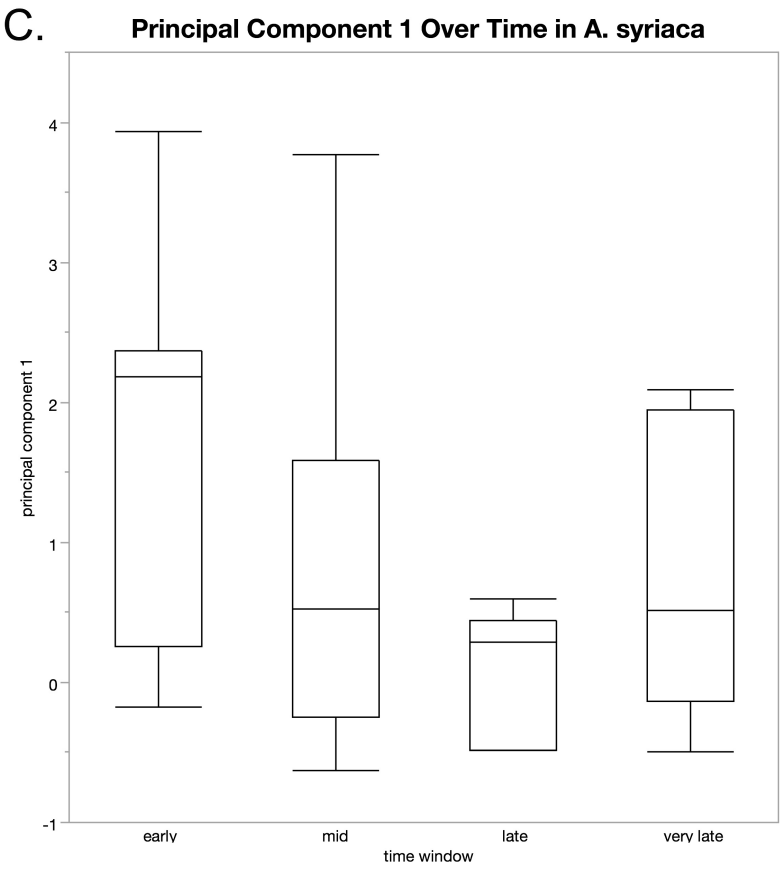
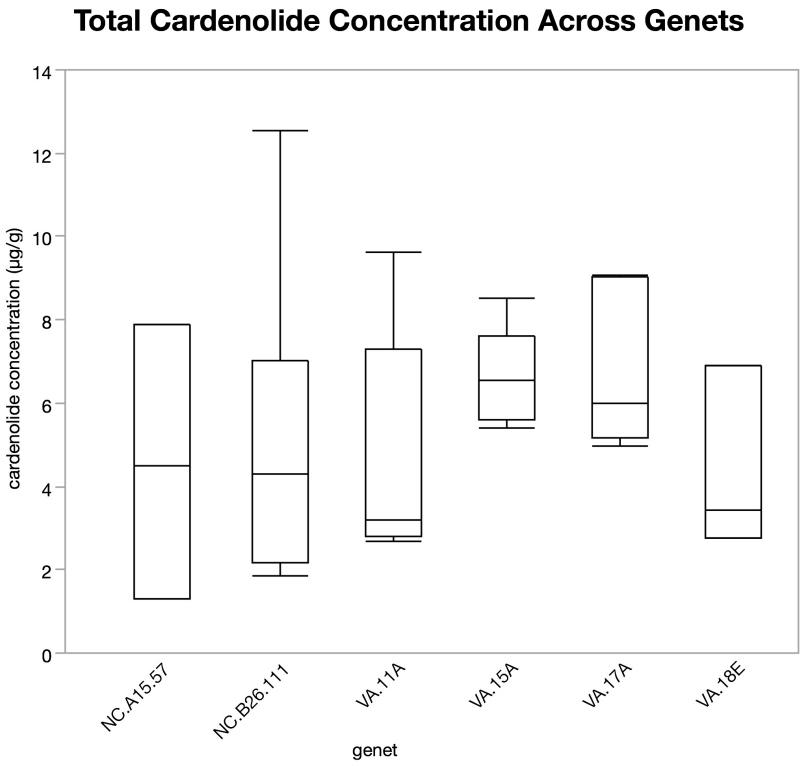


Figure 7: A. Comparison of principal component 1 values for all data between 'early', 'mid', 'late' and 'very late' time windows. 'Time Window' helps to explain principal component 1($F(3, 58)=12.18$, $p<0.0001$). **B.** Comparison of principal component 1 values for insect samples between 'early', 'mid', 'late' and 'very late' time windows. 'Time Window' helps to explain principal component 1 in insects ($F(2, 22)=10.05$, $p=0.0009$). **C.** Comparison of principal component 1 values for plant samples between 'early', 'mid', 'late' and 'very late' time windows. 'Time Window' does not help to explain principal component 1 variation in plants. Insect data appear to be driving the variation explained by principal component 1 ($F(3, 29)=1.85$, $p=0.1628$).

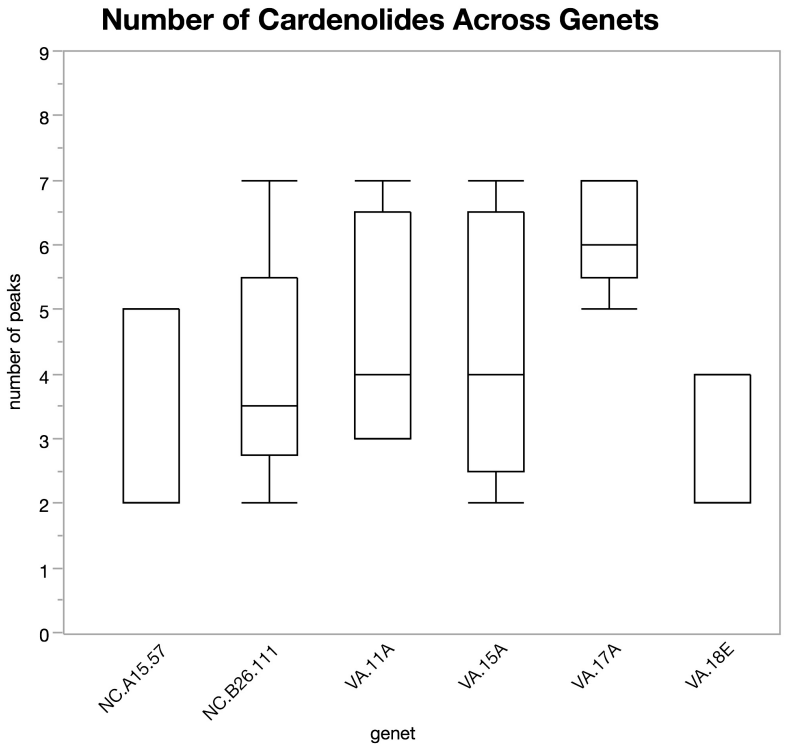
Variation in Cardenolide Content Between Milkweed Food Plants

While PCA did not indicate that variation among all cardenolide concentration values might have a basis in the milkweed genet those chemicals originally came from, we sought to understand just how different the various genets were from one another. We show that no single genet's tissue possessed a higher total cardenolide concentration than another ($F(6, 29)=0.62$, $p=0.71$; Figure 8a). We

A.


Total Cardenolide Concentration Across Genets
cardenolide concentration (µg/g)
0
2
4
6
8
10
12
14
NC.A15.57
NC.B26.111
VA.11A
VA.15A
VA.17A
VA.18E
genet

B.


Number of Cardenolides Across Genets
number of peaks
0
1
2
3
4
5
6
7
8
9
NC.A15.57
NC.B26.111
VA.11A
VA.15A
VA.17A
VA.18E
genet

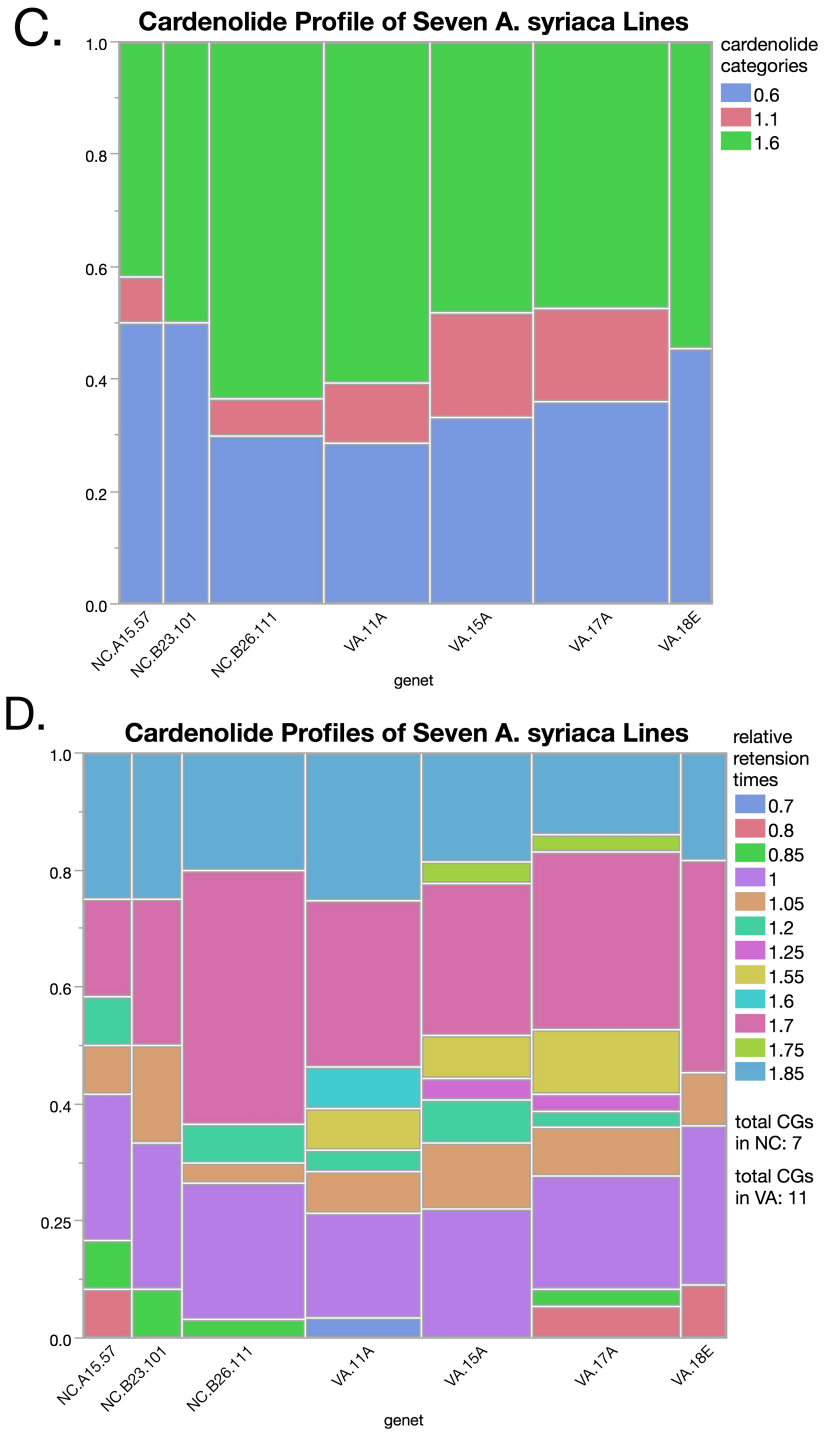


Figure 8: A. The total cardenolide concentrations of the seven milkweed genets used in the experiment. We observed no difference ($F(6, 29)=0.62$, $p=0.71$). **B.** The total number of cardenolide peaks observed in the seven milkweed genets. Their difference did not meet our significance threshold ($F(6, 29)=2.45$, $p=0.056$; Figure 7b). **C.** A statistical comparison of cardenolide profiles between genets showed them to not be meaningfully different ($c^2(12, n=156)=11.61$, $p=0.47$). **D.** The cardenolide profiles of each of the seven milkweed genets sampled in the experiment. The plot shows which cardenolides (by relative retention time) were present in each of the plant lines and how frequently it appeared in samples from those individuals

found the variation in the number of cardenolides between genets to be just below our significance threshold of $\alpha=0.05$ ($F(6, 29)=2.45$, $p=0.056$; Figure 8b). No difference was observed between the suites of individual cardenolides expressed by the seven milkweed lines ($c^2(12, n=156)=11.61$, $p=0.47$; Figure 8c).

Variation in Cardenolide Concentration Across Tissues

Having demonstrated so far that cardenolide concentrations could only be said to vary as a function of the species those chemicals were found in, we sought to understand the effect of our samples' tissue types upon this variation. We showed that variation in principal component 1 (but not 2) could be explained by differences between the tissue types, "leaf", "frass", "larvae", "pupae" and "adults" ($F(4, 58)=12.14$, $p<0.0001$; Figure 9). Comparisons of individual tissue types reveals that leaf tissue is significantly different from frass, larvae, pupae and adults ($t(54)=4.43$, $p<0.0001$; $t(54)=5.13$, $p<0.0001$; $t(54)=4.28$, $p<0.0001$; $t(54)=3.08$, $p=0.0016$; respectively). These insect tissue types were not different from one another with respect to principal component 1, leaving 'organism' as the factor that appears to best explain variation in concentration data. Comparison of actual cardenolide concentrations yielded the same trend across tissues. *Syriaca* cardenolides were significantly more concentrated than those of all insect and other tissues - frass, larvae, pupae and adults ($F(4, 288)=6.34$, $p<0.00010$). Insect tissues again were not significantly different from one another.

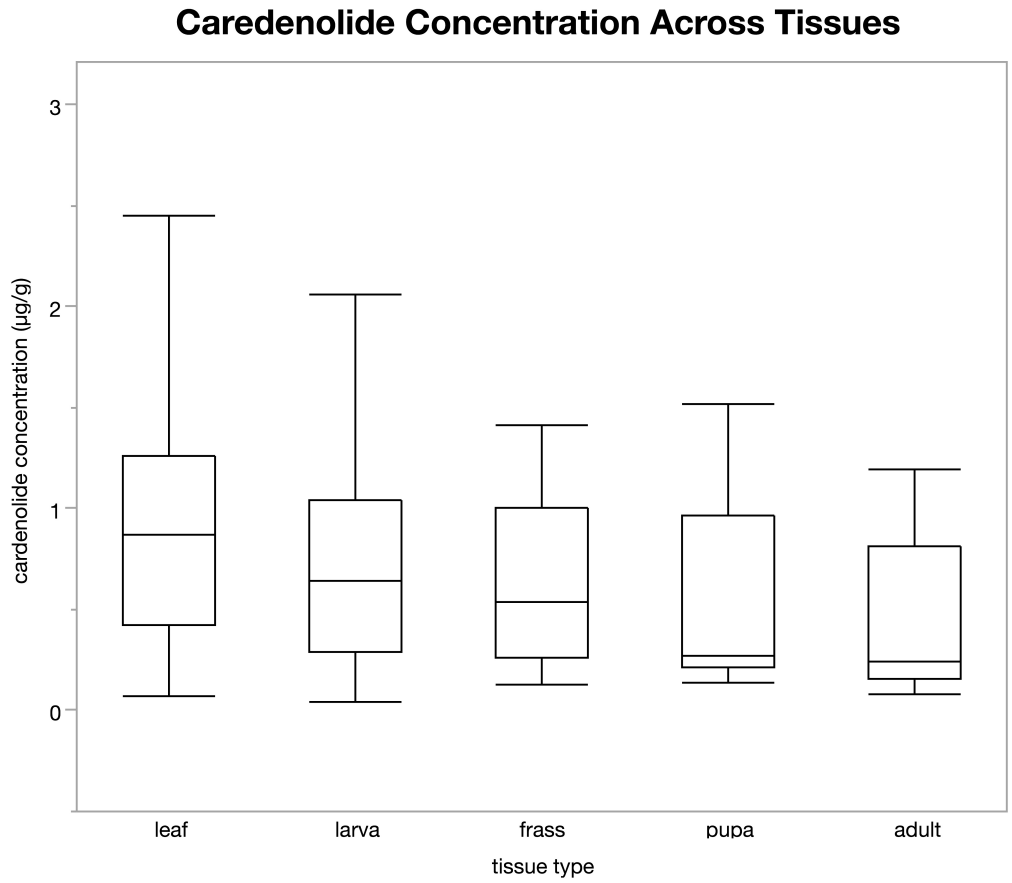


Figure 9: A comparison of cardenolide concentrations for each of the tissue types collected. *Syriaca* tissue demonstrates significantly greater concentration than all others which are not significantly different from another ($F(4, 58)=12.14$, $p<0.0001$; p=.0023, p=.0026, p=.0027, p=.0006).

Variation in 'Percent Contribution' Between A. syriaca *and* D. plexippus

We repeated the PCA described under 'Variation in the Most Common Cardenolides', this time considering the 'percent contribution' of each cardenolide in each sample. This value is the percentage of a sample's total cardenolide concentration represented by each single cardenolide peak. It is intended to reflect the relative reliance of an organism or tissue upon that one chemical species for its defense. We performed another analysis with the five most common cardenolide peaks. Variation in the values of principal component 1 is explained quite well by

the organism of the samples (t(47)=10.3, p<0.0001; Figure 10a). Curiously however, when actual percent contribution data were plotted against the organisms they came from, we were unable to confirm that the two groups were significantly different (t(135)=1.19, p=0.88; Figure 10b).

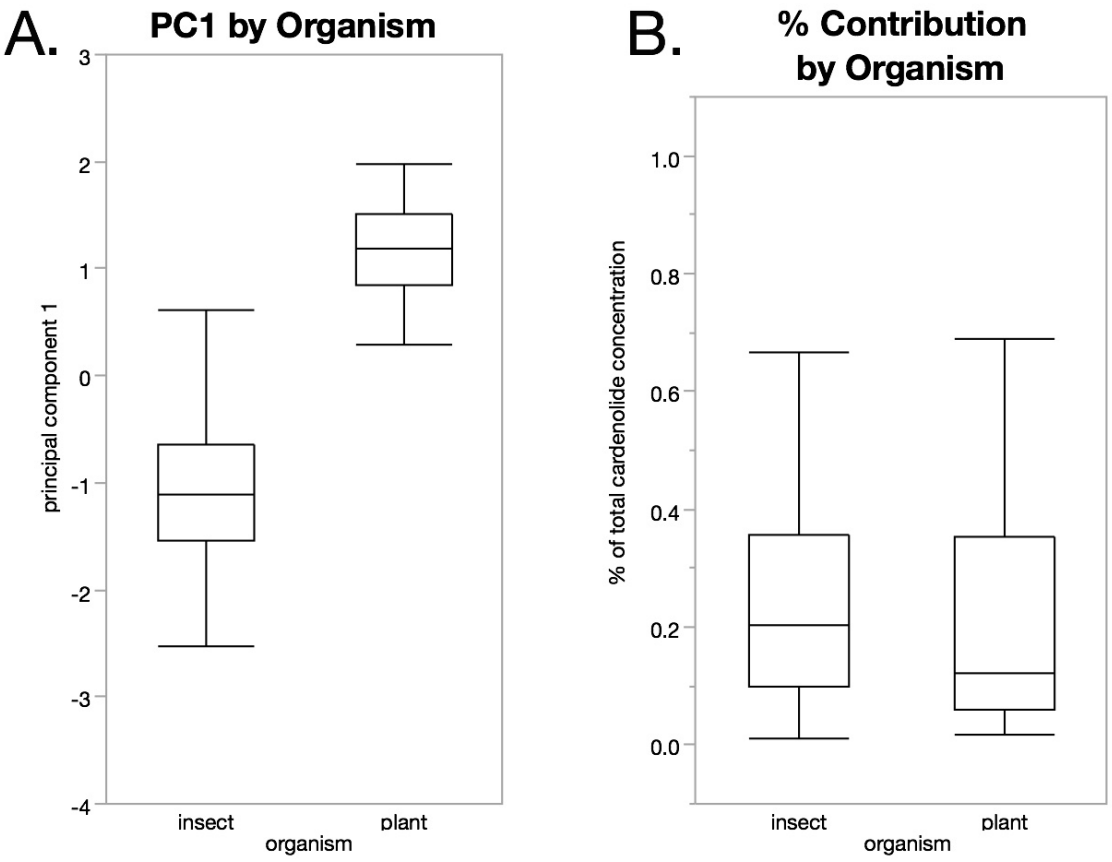


Figure 10: A. A comparison of the first principal component for 'percent contribution' data between organisms (t(47)=10.3, p<0.0001). A strong variation in PC1 values for each group suggests that the differences between the two explain variation in percent contribution data. **B.** A comparison of actual percent contribution data between organisms shows no difference between the two groups, contradicting the results of panel 'A' (t(135)=1.19, p=0.88).

Variation in the Contribution of Individual Cardenolides to Total Concentration

In light of this result, we examined the five most common peaks separately, considering their percent contribution to tissue toxicity in both organisms (Figure 11). The molecule represented by the relative retention time of 1.05 (about 23.1 minutes), present in 36.6% of samples, contributed significantly more to total cardenolide concentration in insects than in plants ($F(1, 17)=8.11$, $p=0.011$; Figure 11a). The cardenolide appearing at a relative retention time of 1.2 (about 26.4 minutes) in 45% of samples also represented a greater percentage of insect concentration than that of the plants ($F(1,20)=9.92$, $p=0.0053$; Figure 11b). The cardenolide appearing at 1.55 (34.1 minutes) was found only in 11.6% samples and only in plant tissue. The cardenolide at 1.7 (37.4 minutes) appeared in 66.6% of samples showed no difference in its percent contribution to the toxicity of insects and plants ($F(1,64)=0.15$, $p=0.69$; Figure 11c). At 1.85 (40.7 minutes), a cardenolide expressed in 56.6% of samples superficially appears to be more important to plant tissue than to insects but the difference falls below our significance threshold ($F(1,34)=1.92$, $p=0.17$; Figure 11d).

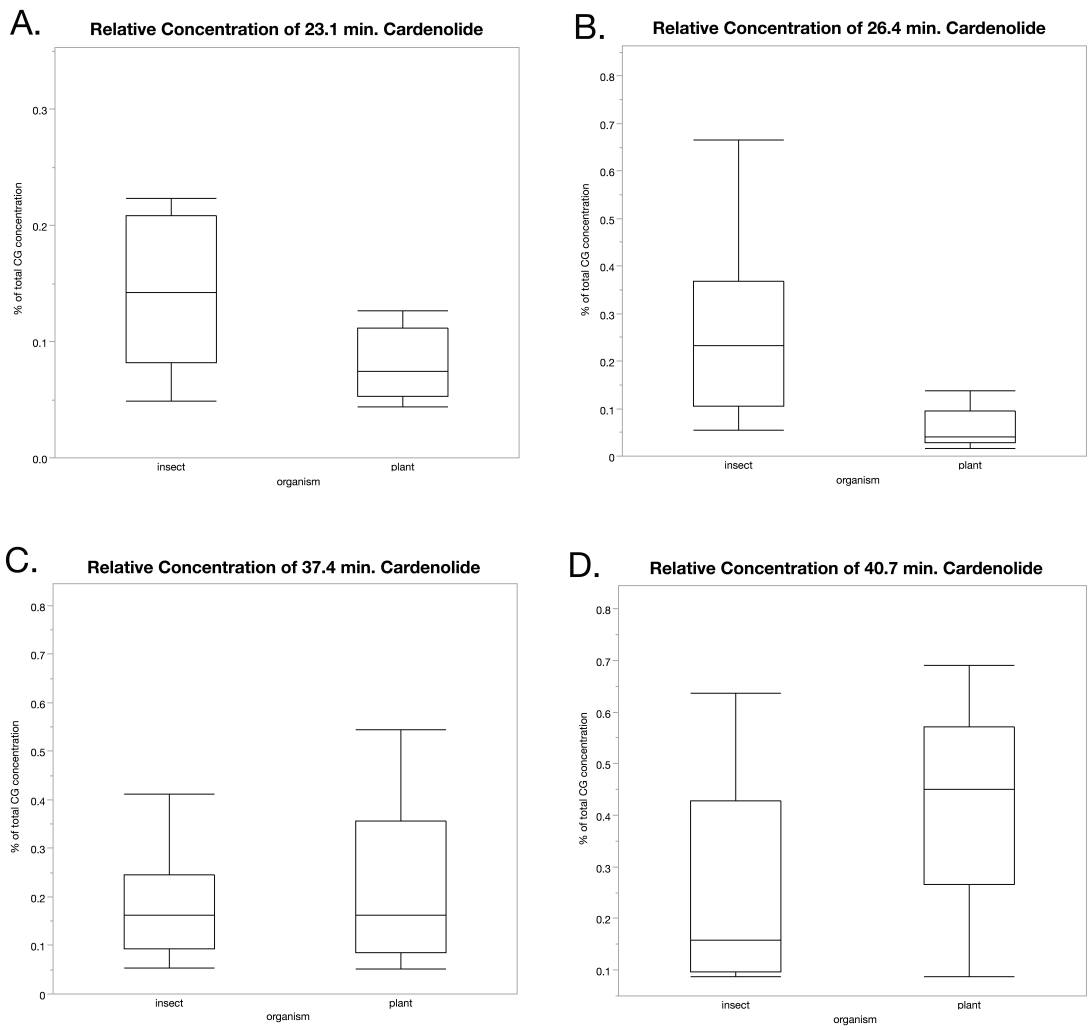


Figure 11: A. The 23.1-minute peak (relative retention time: '1.05-1.1') has a greater contribution to insect cardenolide concentration ($F(1, 17)=8.11$, $p=0.011$). **B.** The 26.4-minute peak (relative retendtion time: '1.2') has a greater contribution to insect cardenolide concentration ($F(1,20)=9.92$, $p=0.0053$). **C.** The 37.4-minute peak (relative retention time: '1.7') does contribute differently to the tissues of milkweed or insects ($F(1,64)=0.15$, $p=0.69$). **D.** The 40.7-minute peak (relative retention time: '1.85-1.9') appears to show a greater contribution in plant tissue, but this distinction is not statistically significant ($F(1,34)=1.92$, $p=0.17$; Figure 10d).

Comparison of 'Cardenolide Profiles' Between Organisms and Tissues

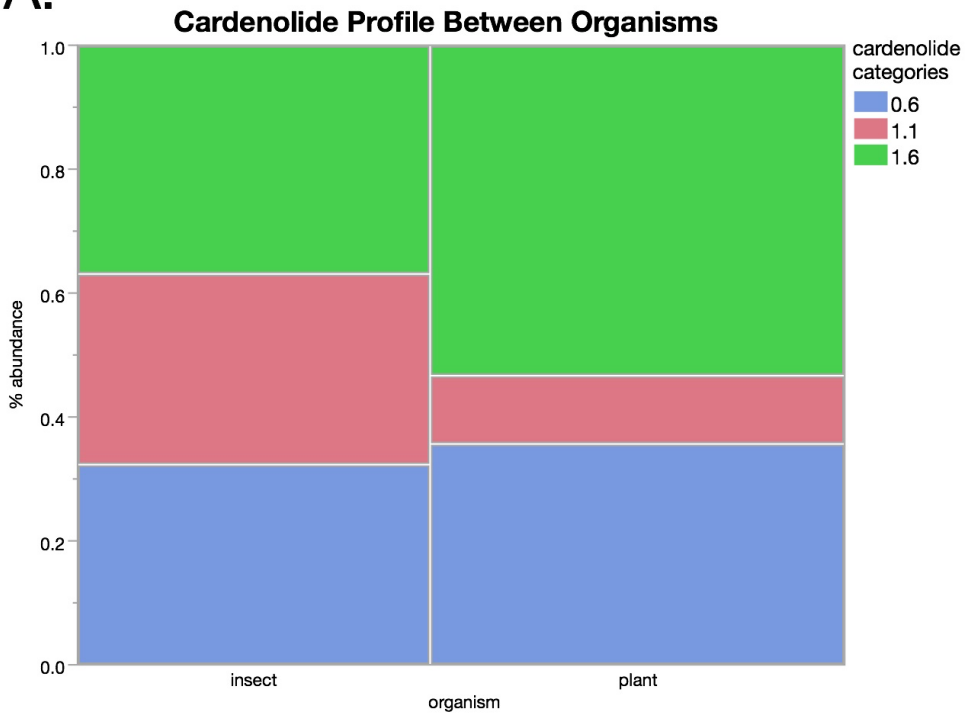
We attempted to compare the 'cardenolide profiles' of samples to determine if their variation could be explained by the factors we've previously identified. A sample's cardenolide profile refers to the range of particular chemical species it possesses and the frequency with which those species appear within a group of like

samples. We compared these profiles using contingency tables confirmed by a Pearson chi-square test. Individual cardenolides were sorted into three categories by retention time, and therefore on the basis of their properties. The first grouping contains the earliest retention times: chemicals of the highest polarity with the smallest molecular weights. The second contains species with intermediate values for each trait and the third set contains the heaviest, least polar cardenolides and the latest retention times. We found the cardenolide profiles of *A. syriaca* tissue and *D. plexippus* tissue to be significantly different ($c^2(2, n=249)=17.9$, $p<0.0001$; Figure 12a). Insect tissue possessed less of the least and most polar cardenolides than plants and possessed more of the intermediate species.

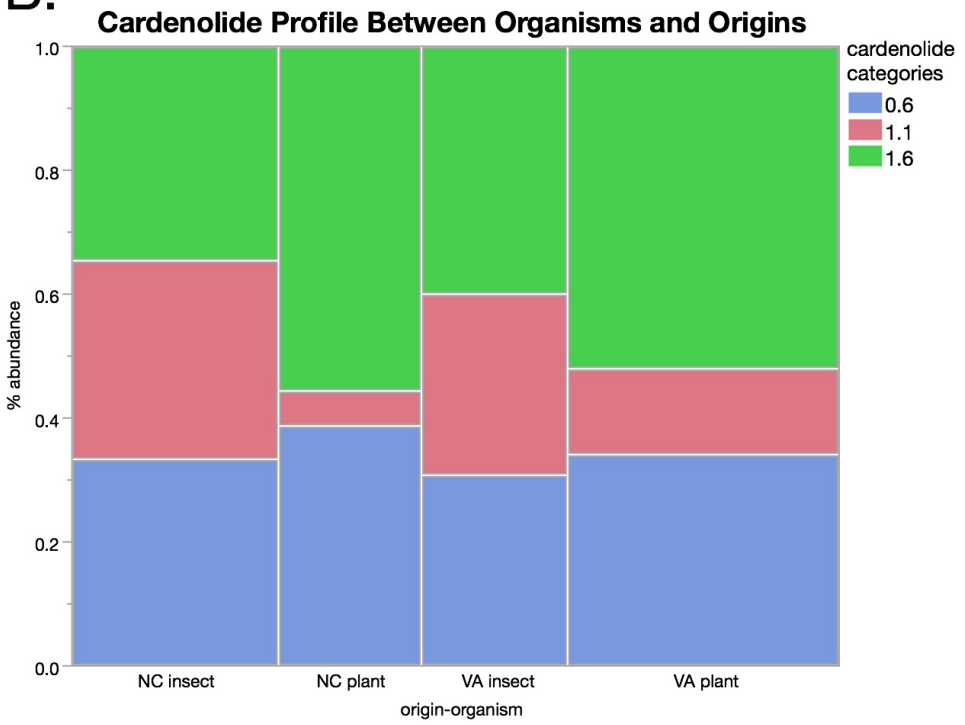
When the tissues of plants and herbivores were sorted by their origins, the resulting groups were found to have significantly different cardenolide profiles ($c^2(6, 249)=19.6$, $p=0.0032$; figure 12b). Between these groups, insects and plants were significantly different from one another, but the separate geographic populations of the insects and plants did not vary significantly from each other. Insects of both origins contain more of the intermediate polarity cardenolides and few of the least polar cardenolides. Plants of both origins on the other hand contain fewer intermediate cardenolides and more of the least polar.

When all tissue types were compared, a chi-square test indicated a significant difference between them ($c^2(8, 289)=24.6$, $p=0.0018$; Figure 12c). Like previous analyses however, this result may be an artifact of leaf tissue's significant difference from all other

A.


Cardenolide Profile Between Organisms
1.0
0.8
0.6
0.4
0.2
0.0
% abundance
insect
plant
organism
cardenolide categories
0.6
1.1
1.6

B.


Cardenolide Profile Between Organisms and Origins
1.0
0.8
0.6
0.4
0.2
0.0
% abundance
NC insect
NC plant
VA insect
VA plant
origin-organism
cardenolide categories
0.6
1.1
1.6

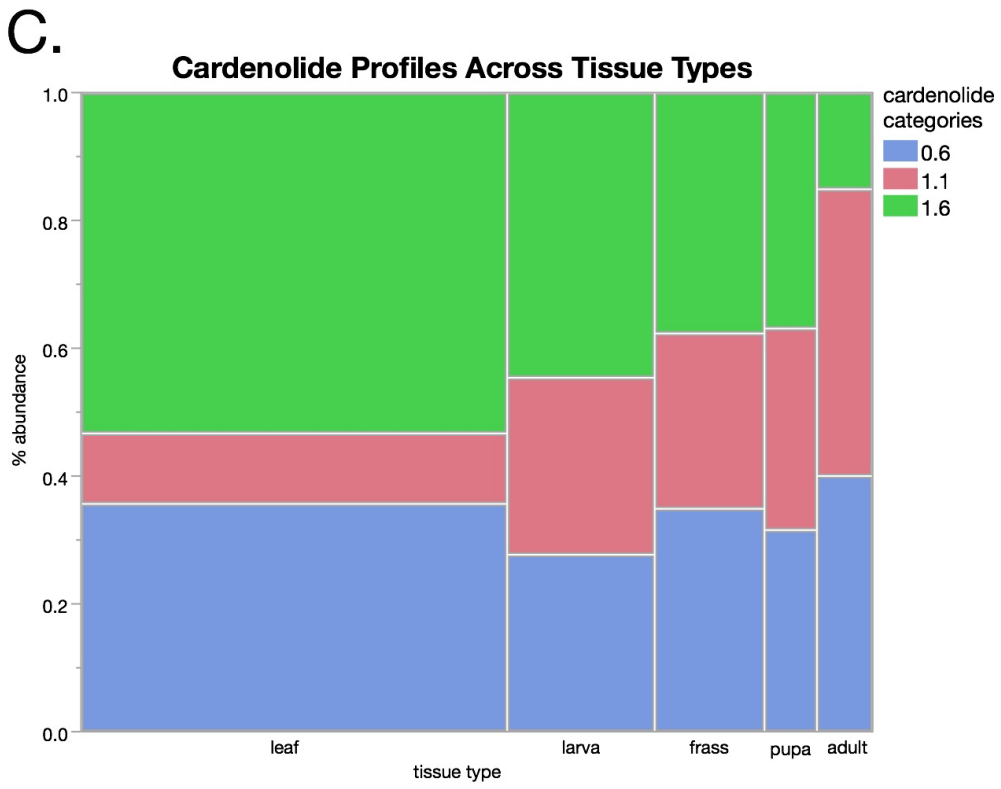


Figure 12: A. A comparison of the cardenolide profiles in Monarch and milkweed samples. The collection of cardenolides employed by the two species for defense are significantly different ($c^2(2, n=249)=17.9, p<0.0001$) **B.** A comparison of the cardenolide profiles in both species after sorting them by their origin. Insect tissue possesses a significantly different profile from plant tissue but within origins plant and insect tissues do not differ from one another ($c^2(6, 249)=19.6, p=0.0032$) **C.** A comparison of the cardenolide profiles in the five tissue types showed a significant difference between them, but this is suspected to reveal the difference between leaf tissue and all other tissues (and thus between plants and insects; $c^2(8, 289)=24.6, p=0.0018$).

tissues – the insect tissues and frass do not appear to differ widely from one another (Figure 12c). However, the nonleaf tissues show a general trend towards decreased prevalence of least polar cardenolides and increased prevalence of most polar cardenolides throughout monarch development.

Cardenolide Profile Variation Over Time

We found that cardenolide profiles across the experiment varied significantly by time ($c^2(6, n=289)=15.7, p=0.015$). Samples collected during the second half of

the experiment contain more of the least polar, late retention time cardenolides, more of the intermediate, middle retention time cardenolides and fewer of the most polar, early retention time cardenolides. Within insect tissue, *D. plexippus* samples did not show any significant fluctuation in their cardenolide profiles over time ($c^2(4, n=93)=5.10$, $p=0.27$; Figure 13a). Likewise, no significant difference in cardenolide profile between time windows was observed in *A. syriaca* samples ($c^2(6, n=156)=7.26$, $p=0.29$; Figure 13b).

Effect of Geographic Origin on Average Cardenolide Concentration

Unable to show variation due to origin itself, we were interested if the results of the concentration analyses were consistent across both regions, North Carolina and Virginia. When divided by the origin of the host plant, North Carolina and Virginia tissue types exhibit the same trend observed with both groups combined. Among the tissues sourced from (or reared on) North Carolina plants, leaf tissue was more toxic than all other tissues ($F(4, 131)=5.25$, $p=0.0006$).

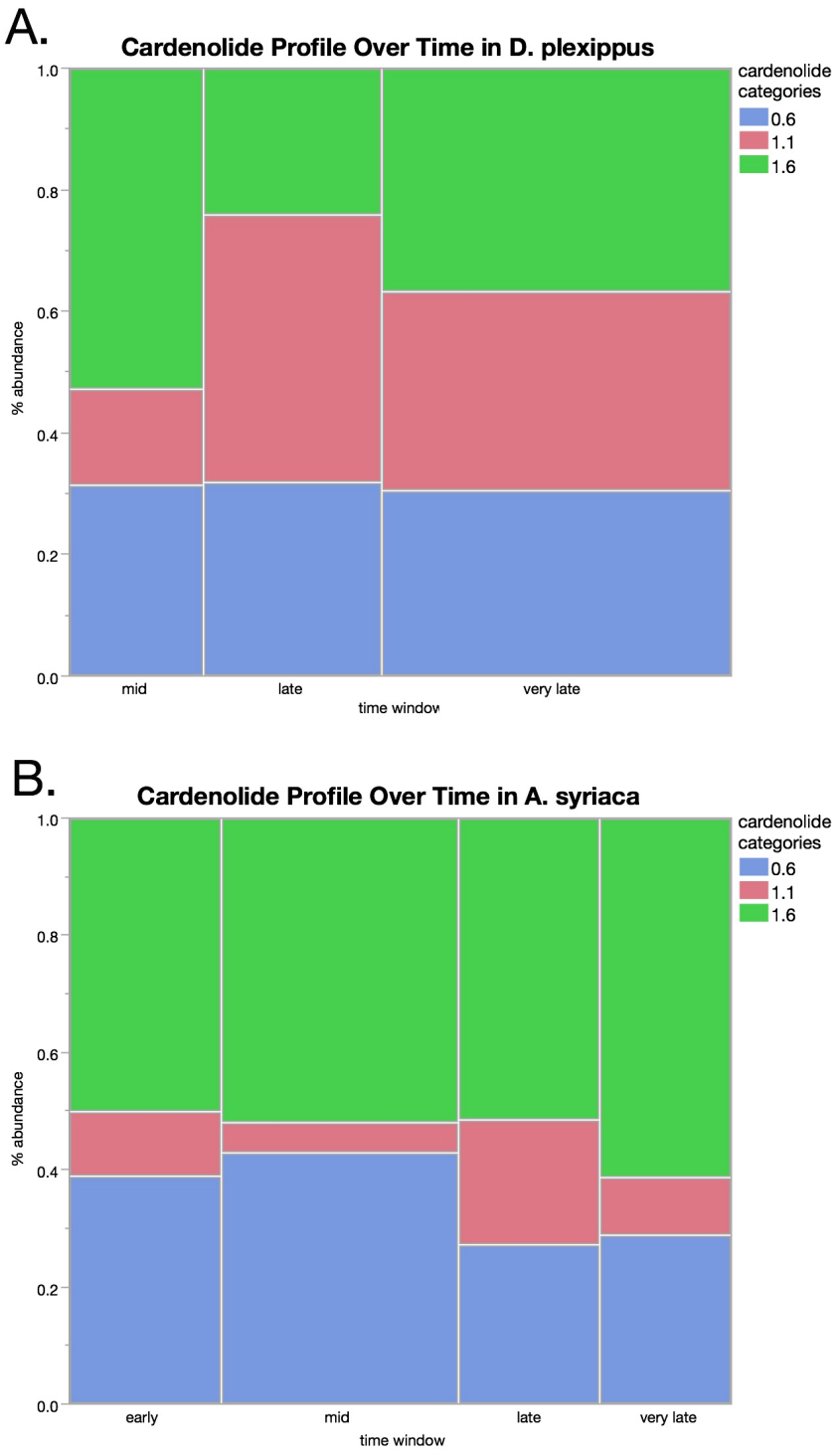


Figure 13: A. Most polar cardenolides (blue), intermediate cardenolides (red) and least polar cardenolides (green) are depicted across time windows**.** Cardenolide profile in insect samples does not vary significantly over time but shows visible differences from plants in the polarities of the cardenolides present ($c^2(4, n=93)=5.10$, $p=0.27$). **B.** Cardenolide profile in milkweed samples does not show significant variation over time ($c^2(6, n=156)=7.26$, $p=0.29$).

North Carolina plants possessed substantially higher average cardenolide concentrations than larval tissue, frass and pupal tissue ($t(127)=2.58$, $p=0.0054$; $t(127)=0.55$, $p=0.0054$; $t(127)=2.69$, $p=0.0081$; respectively). Compared to Monarch adults, the tissue of North Carolina plants was more than twice as toxic, with average concentrations of 0.424 μg/g of tissue and 1.224μg/g, respectively ($t(127)=2.58$, $p<0.0001$). While Monarch larvae, pupae, adults and frass were all significantly different from their food plants, no significant difference was observed between their respective total concentrations within the North Carolina group.

Among Virginia plants, leaf tissue is again the most concentrated source of cardenolides on average ($F(3, 159)=2.82$, $p=0.0408$). The difference in average concentration between plant samples and those from larvae, frass and pupae is less dramatic and the null can be ruled out with less confidence ($t(156)=1.92$, $p=0.027$; $t(156)=1.96$, $p=0.0279$; $t(156)=1.81$, $p=0.035$). Nonetheless, a consistent theme is visible. Again the larval, pupal and frass samples are not significantly different from one another but consistently different from leaves.

Presence or Absence of Individual Cardenolides Between Monarchs and Milkweed

We show that several of the cardenolide peaks identified in this study are unique to plant or insect tissue (Figure 14). Of the 24 distinguishable peaks, 3 were found to be present in both organisms from both locations (1.05, 1.2 and 1.7). 3 cardenolides were unique to insect samples, and shared across both origins (1.1, 1.4 and 2.0). 2 cardenolides were unique to plant samples and shared across both origins (0.8 and 0.85).

Insects raised on North Carolina plants possessed 3 peaks absent in all other tissues while the insects raised on Virginia plants possessed 4 (1.15, 1.3 and 1.9; 1.8, 1.95, 2.15 and 2.2). North Carolina plants did not posses any peaks that could not be found elsewhere while Virginia plants contained 2 (0.7 and 1.55). Insects raised on North Carolina plants did not share any unique cardenolides with their host plants. Insects raised on Virginia plants shared just 1 unique cardenolide with their host plant (1.6).

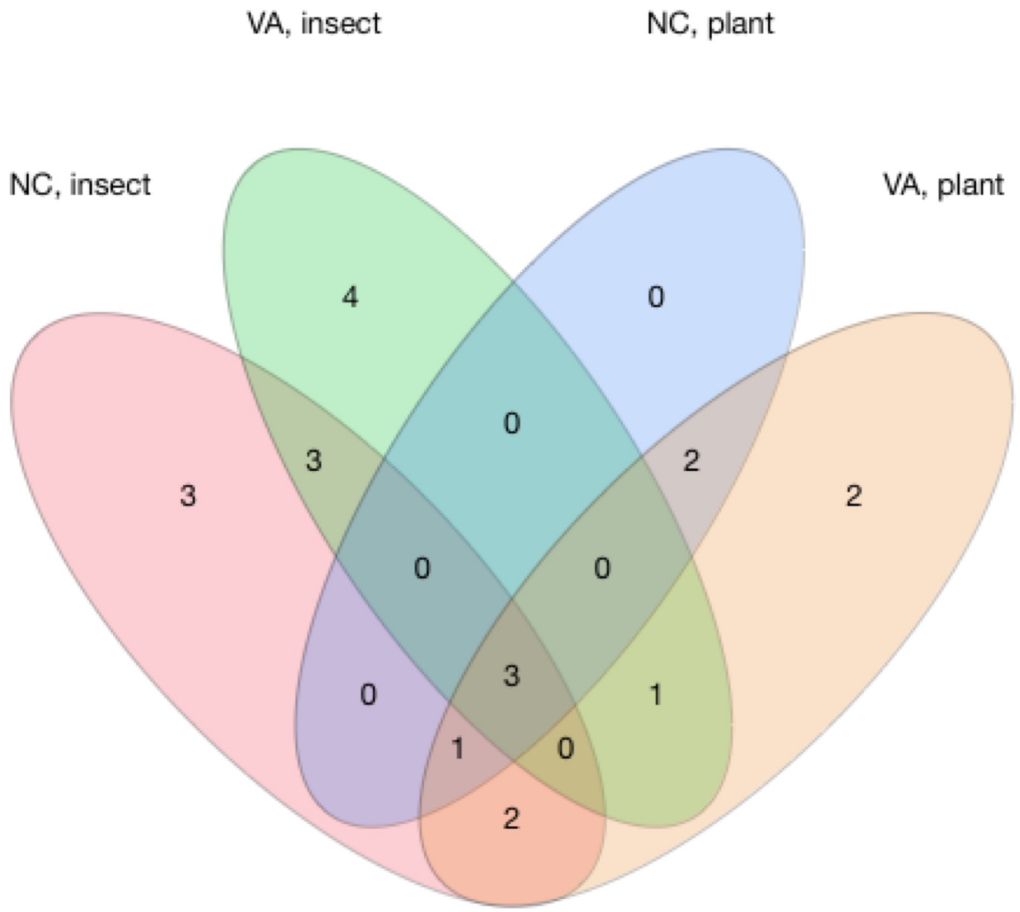


Figure 14: A Venn diagram depicting the overlapping presence of cardenolides between organisms from both origins. Insects possess more unique cardenolides than plants and possess more unique cardenolides between origins than plants.

Cardenolide Concentration Over Time

We found no significant influence of time upon total cardenolide concentration in North Carolina or Virginia plant tissue ($F(6, 11)=4.03$, $p=0.073$; $F(5, 17)=1.50$, $p=0.26$; respectively). This result was unexpected as herbivory has been shown to induce the expression of defensive metabolites in *A. syriaca* (Wang et al., 2014). Any circadian trend in cardenolide expression should have been controlled by the collection of leaf tissue at about 12:00pm each day. We would expect to observe only the effects of herbivory-triggered defense induction over time. It could be that constant Monarch feeding saturated the plant's ability to respond and overwhelmed any underlying circadian behavior. The highest total cardenolide concentrations were observed after about 7 days of feeding in both North Carolina and Virginia plants. Interestingly however, a significant negative correlation was observed between total toxicity in larval tissue and time ($b=-0.89$, $t(1)=2.9$, $p=0.0134$, $r^2=0.230$). Though no significant trendline was obtained for plant tissue of either origin, it should be noted that the highest total concentrations were recorded in larval samples after about seven days at that same time that leaf tissue appeared to be mounting its most robust cardenolide defense.

Effects of Cardenolide Diversity on Total Concentration

'Cardenolide diversity' refers to the total number of cardenolide peaks present in a sample. We found *A. syriaca* tissue to have significantly greater diversity than *D. plexippus* samples ($t(50)=2.77$, $p=0.0039$).

Regression analysis revealed that total cardenolide concentration shows a negative linear relationship with cardenolide diversity in *D. plexippus* samples ($b=-1.19$, $t(1)=6.77$, $p<0.0001$, $r^2=0.142$; Figure 15a). Insect samples that are more poisonous possess fewer cardenolide species. In *A. syriaca* however, a positive linear relationship between toxicity and diversity was observed ($b=0.56$, $t(1)=5.58$, $p<0.0001$, $r^2=0.149$; Figure 15b).

This relationship was investigated across tissue types. A strong positive linear correlation was shown between total concentration and diversity of cardenolides in Monarch adults ($b=0.27$, $t(1)=13.06$, $p<0.0001$, $r^2=.905$). Interestingly, however, the total toxicity of larval tissue showed a negative linear correlation with cardenolide diversity ($b=-1.94$, $t(1)=4.2$, $p<0.0001$, $r^2=.250$). No relationship between total concentration and diversity was shown in leaf samples.

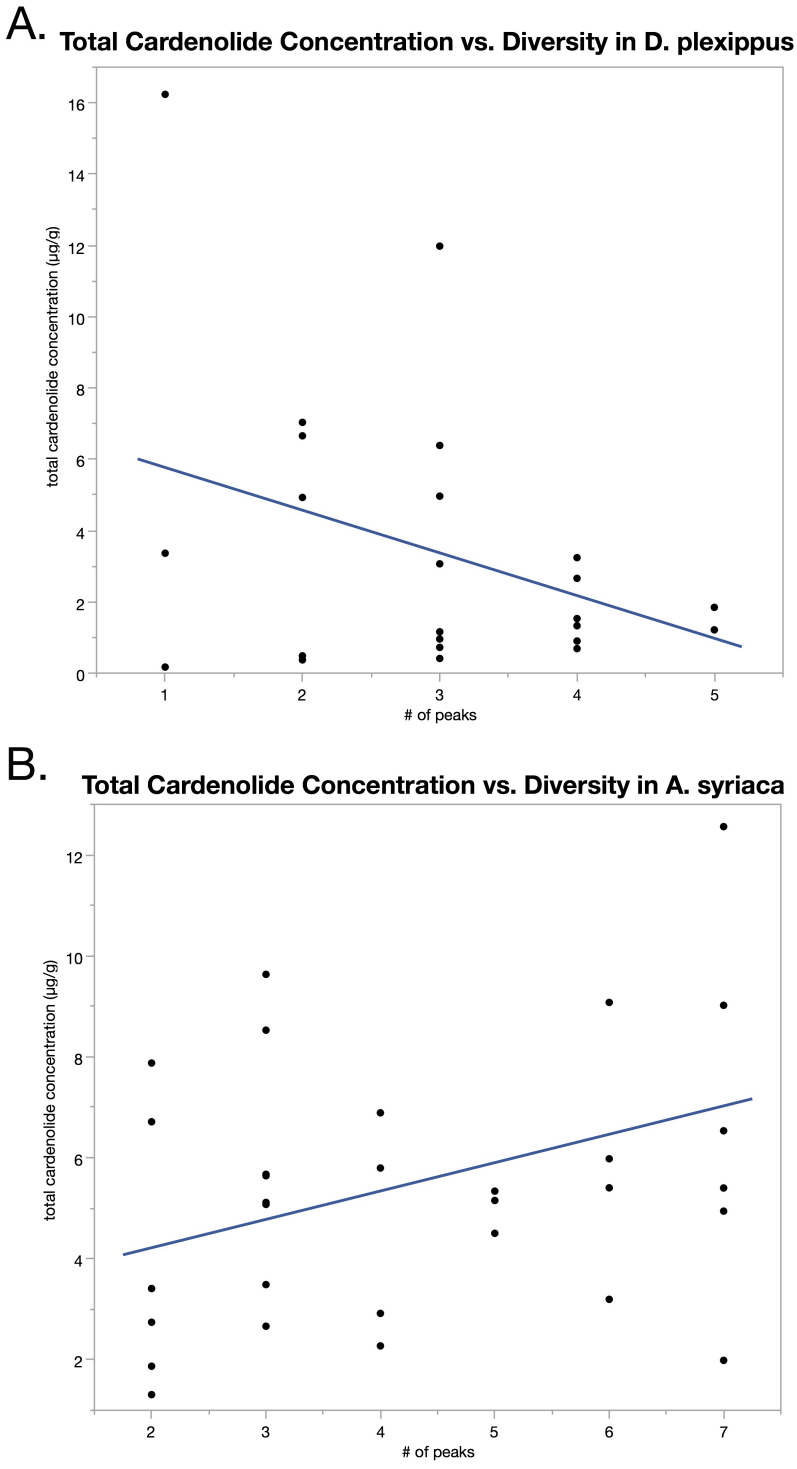


Figure 15: A. A linear regression of total concentration against cardenolide diversity in insect samples. Insect tissues were less toxic with more cardenolides (b=-1.19, t(1)=6.77, p<0.0001, r^2=0.142). **B.** A linear regression of total cardenolide concentration against cardenolide diversity in plant samples. Milkweed tissue grew more toxic with increasing diversity (b=0.56, t(1)=5.58, p<0.0001, r^2=0.149).

Discussion

Despite recent publications alleging no decline in the summer breeding population over the past two decades, concern for dwindling Monarch numbers remains the majority-held opinion in the field. This position is substantiated by evidence of losses at overwintering sites and sagging levels of egg production (Brower et al. 2012; Pleasants and Oberhauser 2013; Pleasants et al. 2016). The methods of Badgett and Dyer, 2015, cited several times in defense of the conclusion that decline is not taking place, have been widely criticized for potentially recounting insects and for collecting data outside of heavy agricultural areas where the species is suffering most severely (Pleasants et al. 2016; Pleasants and Oberhauser, 2013).

We note here that our 'cardenolide criteria' for spectral interpretation are quite conservative. We impose several guidelines not found elsewhere in the literature or in the interpretation protocols referenced. While we may omit some cardenolides, we surely exclude many compounds with similar peaks that would have otherwise been included by the traditional approach. Consequently, some of the discrepancies that arise between our findings and those of previous work may be ascribed to this methodological difference. That said, we can be more confident in the authentic identity of our peaks. We think it better to underestimate the trend than inadvertently invent one with spurious data.

In this study, we are able to show that some cardenolides are more common than others (the peak at 37.4 minutes is most common) and that these chemicals

appear at widely varying concentrations. We show that Monarch and milkweed tissue have significantly different cardenolide content.

We can say that the Monarch butterfly's sequestration of defense chemicals appears to take place with some discrimination. We show here that defensive chemicals appear at lower concentrations in insect tissue than in the plants they came from. The cardenolide profiles of insect and plant tissue are significantly different – butterflies and milkweed plants do not defend themselves with the same compounds. Monarchs also contain fewer cardenolides than plants. Cardenolide sequestration in the Monarch butterfly appears to reject some chemicals in favor of others.

We observed the presence of cardenolides in insect samples that were not found in plants. We infer from this observation that the Monarch may be converting some plant-sourced cardenolides into new chemical species, revealed by unique retention times. These novel peaks appear at retention times that reflect the higher polarity cardenolides which insects rely upon most heavily (Malcolm and Brower, 1989; Lynch and Martin, 1987). We observe novel cardenolide peaks in the 'most polar' and 'intermediate' peak categories and we've shown a reduced prevalence of 'least polar' peaks in insects. The presence of more polar cardenolides in insects than plants conforms to previous observations in the literature (Malcolm and Brower, 1989; Lynch and Martin, 1987). We speculate that conversion in the butterfly may be a selectively advantageous pathway if less valuable cardenolides are being retrofitted into those structures with the greatest defensive utility. The most polar cardenolides are known to be the most toxic (Malcolm et al., 1987).

From our observation of a positive linear relationship between total cardenolide concentration and cardenolide diversity in *A. syriaca* we conclude that increasing leaf tissue toxicity appears to require the addition of new chemical species, not merely the upregulated production of existing ones.

To address one of the primary objectives of this investigation: the separate life stages of the butterfly do not differ in their possession of defensive chemicals. We find that the Monarch butterfly's cardenolide profile, average and total cardenolide concentration, and cardenolide diversity to be stable throughout its development. This contradicts our observation that cardenolide concentration variation can be explained by time window when all tissues are considered simultaneously. We suspect that this implies differences in the concentration of individual cardenolides over time that are not visible within the Monarch's life stages individually.

After combining separate retention times into several small groups, we found that this strategy does not enable us to explain the same variation we were able to with individual cardenolides. This leads us to wonder if cardenolide discrimination in sequestration cannot be easily explained by their general properties. Perhaps the process is more complicated (or arbitrary) than the exclusion of some excessively non-polar structures.

It has been shown that microgeographic variation in populations of a milkweed species can influence the cardenolide content of the Queen butterflies that feed on them as larvae (Moranz and Brower, 1998). While geographic differences were unable to explain substantial variation in our cardenolide data, we suspect that

with a greater number of North American populations of *A. syriaca* represented we might be able to visualize the same trend in *D. plexippus* for the first time.

We show that plant cardenolide concentration does not vary over time while concentration in insect tissue progressively declines. We propose that cardenolides may not be especially stable in Monarch tissue and that larvae sequestering these compounds eventually achieve a threshold beyond which they are no longer accumulating new stores, but replenishing those that are lost. As the insects consume plant tissue, they accumulate body mass faster than they replenish their chemical defenses, giving decline in total concentration over time.

Future Directions

Many of the statistical analyses employed here were constrained by the size of our dataset. Replications of this experiment or a similar development-spanning feeding trial would enable several of these same analyses to be performed with greater precision. With sufficient data collection it might be possible to consider and compare cardenolide profiles by their individual cardenolide peaks, instead of by broad classifications of those peaks. In fact, such precision may be necessary in concentration analyses: we observed broadened retention time bins to be of limited usefulness in comparing cardenolide concentrations by properties.

Replication of this experiment with *A. syriaca* individuals from geographically disparate populations might be able to reveal geographic cardenolide variation in plants and insects that we were unable to simulate here. Specifically, the inclusion of multiple North American regions, especially with

latitudinal variation, would help to clarify two essential facets of the Milkweed-Monarch relationship. The ecology lab of our vital collaborator, Harmony Dalgleish, has recently completed the collection of *A. syriaca* rhizome specimens from across North America. We propose a replication of this experiment to further refine its methods and include additional data to further our exciting collaboration.

In further investigations of this phenomenon, it is essential that we obtain mass spectrometry data for our compounds of interest to validate our conservative approach to spectral interpretation. Even more importantly, mass spectrometry gives us the opportunity to learn the identity of peaks that do not fit our current criteria and expand them accordingly if necessary. Obtaining the structures of these compounds also gives us the ability to make even more detailed observations about the unifying molecular characteristics of the chemicals unique to plants or insects. Generalizations could be made on the basis of geometry or reactivity instead of on the basis of molecular weight and polarity alone.

An article published in 2007 by De Roode and colleagues provides a protocol for quantifying the virulence and spore load of an alveolate parasite *O. elektroscirrha* in the bodies of adult Monarchs. A paper published by De Roode in 2016 compared the virulence measures of Monarchs fed two *Asclepias* species with radically different cardenolide content, *incarnate* and *curassavica*. They show that host plant species contributes significantly to parasite infection, replication and virulence. We suggest a replication of the developmental feeding trial outlined here using several geographic varieties of one species, *A. syriaca*, where De Roode's virulence-calculating protocol is employed to compare parasite load in adult Monarchs.

Because the spore of the parasite are passed from mother to offspring during oviposition, the results of such an experiment would permit conclusions regarding the selective advantage of dining on (and sequestering) the cardenolides of one region's milkweed compared with another.

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